SIMULTANEOUS QUANTIFICATION OF FIRST-LINE ANTI-TUBERCULOSIS DRUGS AND METABOLITES IN HUMAN PLASMA

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I. DECLARATION

I, **Marian Tafadzwa Mazanhanga,** hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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II. ABSTRACT

Tuberculosis (TB) currently kills more people than any other infectious disease worldwide, the highest burden being in Africa and Asia (1). Therapy recommended for drug sensitive TB consists of a cocktail of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB), all given in a 2-month intensive phase, followed by only INH and RIF in a 4-month continuation phase. Clinical studies seeking to optimize dosing, gain more knowledge on the pharmacokinetics and pharmacodynamics of the drugs and compare current therapy to alternative regimens are required (2, 3). Therapeutic drug monitoring (TDM) is frequently carried out in cases responding poorly to therapy (3, 4). Both clinical studies and TDM require bioanalytical methods for quantifying drug concentrations in biological fluids. Several methods have been developed, mostly analysing individual drugs but a few analyse combinations. Ideally, quantification of all four drugs in one method is desirable as it is economical and allows high throughput.

A method was developed and validated for the quantification of first line anti-tuberculosis drugs EMB, INH, PZA and RIF and the metabolites N-acetyl isoniazid (AcINH) and 25-desacetyl rifampicin (desRIF). Sample preparation consisted of protein precipitation, followed by high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection. Deuterated internal standards for each analyte (AcINH-d4, desRIF-d3, EMB-d4, INH-d4, PZA-15N,d3 and RIF-d3) were used. Mean recoveries of the analytes from plasma were as follows: AcINH 106.5%, DesRIF 123.2%, EMB 105.3%, INH 110.1%, PZA 132.0% and RIF 127.7%. Sample preparation was followed by reverse phase liquid chromatography on an Agilent 1200 series HPLC system using an Agilent Poroshell 120 EC- C18 2.7µm, 4.6 X

50mm analytical column. Separation of all analytes was achieved using a mobile phase gradient consisting of an aqueous mobile phase A (0.05% formic acid in water) and an organic mobile phase B (0.05% formic acid in a mixture of methanol and acetonitrile, 1:1). A T-junction splitter was used to reduce the mobile phase flow to the ion source by about 30%. Retention times for AcINH, desRIF, EMB, INH, PZA and RIF were 2.45, 5.40, 1.75, 2.22, 4.30 and 5.68 minutes respectively.

An AB Sciex API 4000 triple quadrupole mass spectrometer at unit mass resolution in the multiple reaction monitoring (MRM) mode was used for detection, monitoring the following transitions for the six analytes: AcINH 180 → 121, desRIF 784 → 752, EMB 205 → 116, INH 138 → 79, PZA 124 → 81 and RIF 823 → 792. An electrospray ionisation (ESI) source in the positive ion mode was used to couple the mass spectrometer to the LC system.

Accuracy and precision were assessed over three consecutive and independent runs. The calibration curves fit quadratic regressions for all analytes, with weighting of 1/x (where x=concentration) for all except PZA which had a weighting of $1/x^2$ over the calibration range. Calibration ranges in µg/ml were as follows: AcINH 0.050 – 12.5, desRIF 0.040 – 10.0, EMB 0.020 – 5.00, INH 0.100 – 25.0, PZA 0.32. – 80.0 and RIF 0.120 – 30.0, based on peak area ratios.

A 1:4 dilution of the QC Dilute sample showed that concentrations of up to 20.0 µg/ml for AcINH, 16.0 µg/ml for desRIF, 8.00 µg/ml for EMB, 40.0 µg/ml for INH, 128 µg/ml for PZA and 48.0 µg/ml for RIF in plasma could be analysed reliably when diluted into the calibration range. No significant carry-over was observed for all analytes.

The method was shown to be reproducible when human plasma samples from six different sources were analysed and endogenous matrix components had no significant effect on the assay. All analytes were stable in plasma for at least four hours on ice, and when subjected to three freeze-thaw cycles. Reinjection reproducibility experiments showed that all analytes except PZA could be reliably analysed by re-injecting an entire batch after about 48 hours. Quantification of AcINH, INH and RIF was not significantly affected by 2% hemolysis of sample while desRIF, EMB and PZA were significantly affected. Data was analysed using Analyst ® version 1.6.2 software.

With wide calibration ranges, the assay is suitable for both routine TDM and PK studies. Concurrent analysis of metabolites allows inferences to be made on the PK of the two main TB drugs. The total run time of 6.5 minutes per sample combined with the simple sample preparation procedure, the method is more economical on both time and resources than single analyte assays.

III. ACKNOWLEDGEMENTS

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Above all, I thank God Almighty, for sustaining me, giving me strength of body and mind throughout the process of attaining this degree.

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LIST OF ABBREVIATIONS

µg- microgram

µl- microlitre

AcINH- Acetyl isoniazid

ADR- Adverse drug reaction

CAD- Collision gas

CE- Collision energy

CEP- Collision cell entrance potential

CUR- Curtain gas

CXP- Collision cell exit potential

desRIF- 25-Desacetylrifampicin

DMSO- Dimethylsulphoxide

DP- De-clustering potential

EMB- Ethambutol

EP- Entrance potential

ESI- Electrospray Ionisation

FP- Focussing potential

HPLC- High Performance Liquid Chromatography

INH- Isoniazid

IS- Internal Standard

IS voltage- Ionspray voltage

LC-MS/MS- Liquid chromatography tandem mass spectrometry

MRM- Multiple reaction monitoring

MS- Mass spectrometry/er

MTB- *Mycobacterium tuberculosis*

PD- Pharmacodynamics

PK- Pharmacokinetics

PZA- Pyrazinamide

RIF- Rifampicin

TB- Tuberculosis

TDM- Therapeutic drug monitoring

TEM- Temperature

WHO-

World Health Organization

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Chapter 1 : INTRODUCTION

The age-old disease continues to threaten human health across the globe, but more so in less developed countries, particularly in Africa and Asia. Tuberculosis, according to the World Health Organization (WHO), kills more people than any other infection yearly (1). Several drugs are available for treating the disease and with proper management, treatment is highly successful. The currently preferred regimen for the treatment of new cases of drug susceptible tuberculosis (TB) includes four drugs: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB). These are commonly referred to as the 1st line TB drugs. All four drugs are administered in the initial 2-month intensive phase, followed by a 4-month long continuation phase with INH and RIF only. Multi-drug therapy has been shown to reduce the emergence of resistant mycobacterial strains and gives better efficacy (5). The four drugs act at different target sites, which may result in additive effects or synergy among them. In addition, if a pathogen develops resistance to one drug it is likely to be killed by the other drugs due to activity at different sites from that at which the ineffective drug acts. Although more effective, multi-drug therapy may subject patients to more adverse drug effects through drug-drug interactions, hence adequate patient monitoring is essential. Therapeutic drug monitoring (TDM) is one of the strategies that are used to monitor patients. Although it has a limited role in many settings in the treatment of TB, perhaps because treatment is highly successful and TDM may be expensive (6), there are cases where TDM is called for, such as poor or slow response to treatment and suspected adverse drug reactions.

Although all the 1st line drugs were introduced more than 50 years ago, many gaps still exist in what is known about their pharmacodynamics (PD) and pharmacokinetics (PK). Several studies have shown that drug concentrations are frequently below or above the target

concentrations (7-9) and questions arise on whether this is due to inappropriate dosing, poor formulation of medicines or to patient related factors such as age, pharmacogenetics and co-morbidities. In some cases, drug metabolites play a role in the efficacy and/or toxicity of drugs, hence these may need to be monitored. Clinical studies are necessary, and continue to be carried out, to establish optimum dosing which will result in high efficacy with minimum adverse drug reactions (ADRs). These studies may also help to establish PK-PD relationships, to identify poorly formulated medicines and to compare the existing regimens to new ones.

Both TDM and PK-PD studies require reliable and accurate methods for the quantification of drugs in biological matrices. The most commonly used matrices include blood, plasma, serum and urine. These matrices require some level of sample preparation prior to analysis. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is one of the most commonly used techniques in bioanalysis as it is highly sensitive and selective. Numerous methods have been developed for the analysis of 1st line TB drugs, many of them looking at individual drugs (10-13) and some analysing combinations (9, 14-17). A few have been developed for the quantification of all four (18-20), each method being unique to other methods. Analysis of all 4 drugs in one method is desirable because it allows economical quantification of all drugs and allows high throughput. However, there are challenges in developing such methods because of the differences in the physicochemical properties of the drugs; it is difficult to reliably analyse molecules of such wide-ranging chemical structures, polarities and stability under the same conditions. Molecular structures and masses of 1st-line TB drugs are show in Figure 1.1. After development, validation is necessary to prove that the method can reliably measure what it purports to measure. Validation involves a set of

experiments under various conditions to measure important parameters such as accuracy, reproducibility and stability.

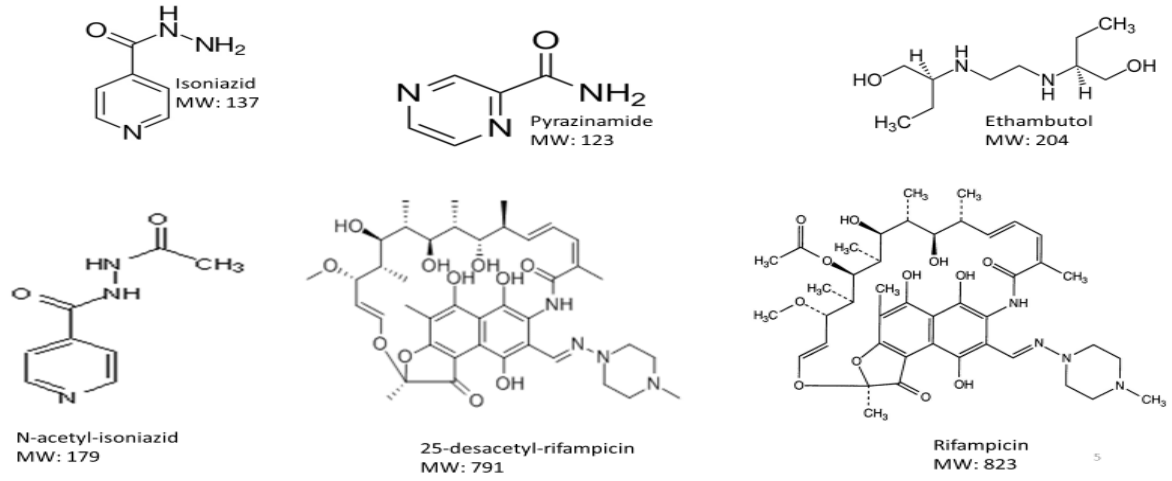

Figure 1.1: Molecular structures and masses of 1st line TB drugs and two metabolites

Although new drugs have been developed, and some relatively old ones re-purposed for TB treatment, the 1st line drugs remain highly effective and better tolerated, hence they are the preferred treatment of choice. The rise in drug resistance combined with the expensive and slow rate of new drug development necessitates good stewardship of the highly successful, well tolerated and more affordable 1st line therapy through means such as TDM and establishing PK-PD relationships. For these purposes, a simple, rapid and robust method was developed and validated to accurately and consistently quantify the 1st line anti-TB drugs and metabolites of the two main drugs in human plasma using LC-MS/MS.

Chapter 2 : LITERATURE REVIEW

2.1. Origins of the disease

Tuberculosis (TB) has afflicted mankind for ages and continues to be a leading threat to human health worldwide. Archaeological evidence suggests that the disease dates to pre-historic times (21, 22). Examination of skeletal remains with similar lesions to those diagnostic of spinal TB, and molecular identification of *Mycobacterium tuberculosis* (MTB) DNA in ancient skeletons provide an insight to the origins of the disease. Despite such evidence, the place and time of origin of the disease, the earliest hosts and evolution of the pathogen remain controversial (22). While pre-historic archaeological evidence from Egypt and the Americas dates to about 50 000 years ago, TB documentation is only first seen in classical Greece (480 – 328 BC), where it was called "phthisis" and was well described by Hippocrates (21, 23). Perhaps due to urbanisation and the associated overcrowding during the industrial revolution in Europe, the disease continued to spread and by the early 19th century tuberculosis had reached epidemic levels in much of Europe and North America (21).

Until 1882 the aetiology of the disease was poorly understood. Some believed that it was heritable while others speculated that it was contagious (21, 24). About 40 years after the discovery that TB is caused by MTB, the first anti-TB drugs, para-amino salicylic acid (PAS) and streptomycin (SM), proved useful in curing the disease, followed by other anti-TB drugs in the mid-20th century, leading to a decline in the incidence of the disease (24). Even research in the field of tuberculosis declined as the disease was no longer considered to be a major threat (23). However, the incidence began to increase again with the advent of the

Human Immunodeficiency Virus (HIV) pandemic in the 1980s (25), especially in Africa and Asia, and in 1993 was declared a global health emergency by the World Health Organisation (WHO). Today it is a leading cause of death among infectious diseases.

2.2. Mycobacterium - the pathogen

In 1882, MTB was identified as the causative agent of TB (21, 26). This pathogen, that has evaded the human immune system for millennia, is a large, rod-shaped bacterium, 2-4 μm long and 0.2 – 0.5 μm wide (27, 28). It is an acid-fast bacterium, so called because of the impermeability of the cell wall to certain dyes and stains. Once stained, acid fast bacteria will retain dyes when heated and treated with acidified organic compounds. In addition to the biopolymers peptidoglycan and arabinoglycan, the cell wall contains complex lipids, the bulk of which are mycolic acids which affect the permeability properties of the cell surface and may be involved in the defence mechanisms of the pathogen against the host immune system as well as in drug resistance (29). The cell wall is of paramount importance to the survival of the pathogen and is a target of some TB drugs. Together with seven other mycobacteria (*M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii, M. canetti* and *M. mungi)* it belongs to the *Mycobacterium tuberculosis* complex (30). While some of the other members of the complex are known to cause human disease, MTB is the most significant threat to human health (31). It is an obligate human pathogen, in contrast to the bacilli belonging to the *Mycobacterium avium* complex (MAC) which cause opportunistic infections, that is, they cause infection almost exclusively in immunocompromised individuals and rarely cause disease in healthy humans. Figure 2.1 is a representation of the structure of MTB.

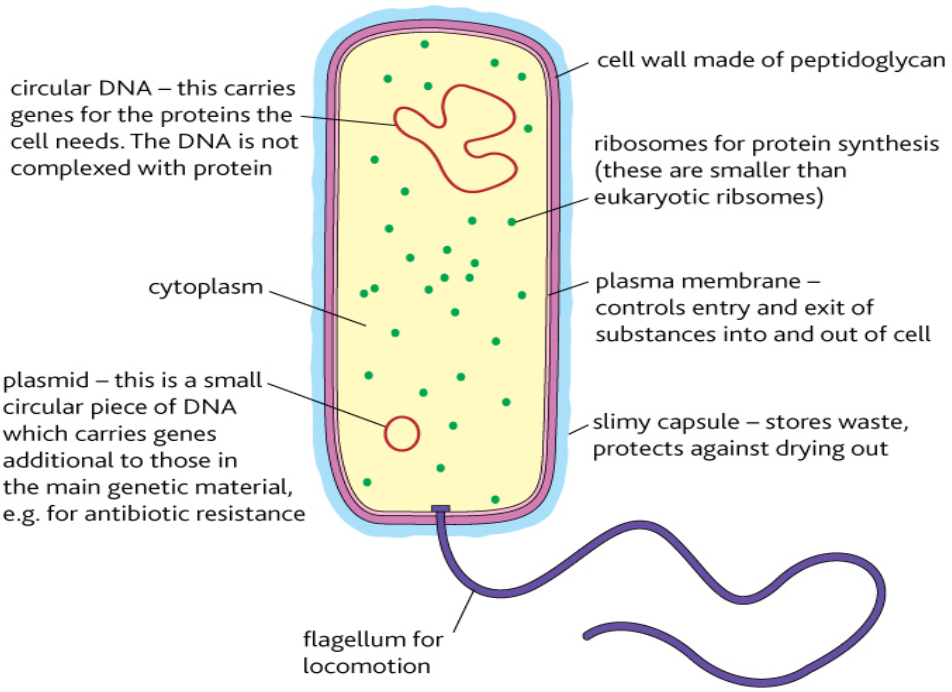

Figure 2.1: Mycobacterium tuberculosis structure (27)

2.3. Epidemiology

About a third of the world's population is thought to be infected by MTB (5). However, this is mostly latent TB which is characterized by having the mycobacterium inside the body without showing or experiencing symptoms. While active TB occurs in all parts of the world, over 95% of cases and deaths are in developing countries (1). South Africa is among the countries with the highest TB burden and was reported to be among the six countries that accounted for 60% of new cases in 2016, together with India, Indonesia, China, Nigeria and Pakistan, and an estimated incidence of 454,000 cases of active TB in 2015 (32). The global incidence is decreasing by about 2% annually but the disease still kills about 1.5 million people every year and has the highest mortality rate among all infectious diseases (1).

Although it is a leading cause of death, TB is both preventable and curable. Cure rates for drug sensitive TB are at least 80% but, without proper TB treatment, about 45% of HIV-negative and most HIV-positive patients will die (1).

Cure rates of drug resistant TB are much lower and may be as low as 22% for extensively drug resistant (XDR) TB. Drug resistance ranges from resistance to a single drug to resistance to many drugs, and is a growing concern, with a prevalence of about half a million worldwide, necessitating the use of second line drugs and in the case of XDR, the use of new drugs. Multi-drug resistant (MDR) TB, by definition, is resistant to the two most commonly used TB drugs isoniazid (INH) and rifampicin (RIF). XDR TB, on the other hand, is resistant not only to INH and RIF but also to fluoroquinolones and at least one second line injectable TB drug (amikacin, capreomycin or kanamycin). Cases resistant to all available TB drugs have been reported and have been termed "totally drug resistant" (TDR) TB (33).

2.4. TB Transmission and pathogenesis

When an infected person coughs, sneezes or talks, droplet nuclei, which can contain 1 – 400 bacilli, are released into the air. Droplet nuclei 1-5 μm in size reach the alveoli when inhaled and MTB bacilli will be taken up mainly by alveolar macrophages as part of normal host defences. Most of the bacilli are killed but some may survive and multiply inside the macrophages (30). These will be released when the macrophage dies, and the mycobacteria may be taken up by new macrophages to start a new cycle, be disseminated to other body parts if they come into contact with the bloodstream or lymphatic system (causing

extrapulmonary TB) or be expelled from the lungs through, for example, coughing with the potential to infect other hosts (34). Various factors interplay in the transmission of TB. These include: the immune status of the exposed person, infectiousness of the person with TB (this depends on the bacterial load of the droplet nuclei produced), the environment (e.g. ventilation) and the exposure level (proximity, duration and frequency). The most common form of the disease occurs in the lungs (pulmonary TB) but it may affect other parts of the body such as the brain, gastrointestinal tract, urogenital tract and bones (extra-pulmonary TB).

Mycobacteria that enter alveolar macrophages initially reside in endocytic vacuoles called phagosomes, which fuse with lysosomes to form phagolysosomes, exposing the pathogens to a hostile environment which is acidic, contains reactive oxygen intermediates, lysosomal enzymes, toxic peptides and possibly reactive nitrogen intermediates (25). Virulent mycobacteria can evade these harsh conditions and continue to multiply inside the host, eventually leading to host cell death. Infected macrophages produce chemokines which attract inactivated monocytes, lymphocytes and neutrophils, but these are not efficient in killing the bacteria. However, by surrounding the infected macrophage, they form granulomatous focal lesions which mitigate the spread of bacteria. Some bacteria can withstand the acidic pH in these oxygen deprived lesions and can survive in a seemingly dormant state, referred to as latent TB infection (25). The mechanism by which these "persistent" bacteria survive in a latent form is not well understood. This latent TB may progress to active TB disease when the host immune system is compromised, as occurs with HIV infection, increasing age and use of certain drugs.

2.5. Clinical manifestation

The signs and symptoms of TB result from the interaction between the host immune system and the pathogen (35). Infected dendritic cells and macrophages produce pro-inflammatory cytokines such as interleukin (IL)-1-α, IL-1-β, tumour necrosis factor (TNF), IL-6, IL-12, and interferon (IFN)-γ, which aid in the T-cell response, promotion of antigen presentation, production of reactive oxygen intermediates (ROI) and activation of phagocytosis (36). The level of immunocompetency of an individual determines the way the disease progresses. Relatively immunocompetent persons usually have latent TB in which they do not manifest clinical symptoms. In about 10% of the population active TB occurs, characterized by symptoms such as fatigue, malaise, weight loss, low-grade fever accompanied by chills and night sweats and coughing which may sometimes produce blood stained sputum. Inflammation in the lung tissue may cause pain and in advanced disease shortness of breath occurs. In some cases, extrapulmonary TB occurs, especially in immunocompromised patients. The most serious cases occur in the central nervous system as TB meningitis or space occupying tuberculomas, both of which have high mortality rates. Disseminated or miliary TB, which can simply be regarded as infection of the bloodstream, is also highly fatal. This can result in multi-organ involvement. Other forms of extra-pulmonary TB include lymphatic (most common form of extra-pulmonary TB), bone, gastrointestinal and genitourinary TB (37).

2.6. Treatment

The introduction of drug therapy changed TB from being a chronic, sometimes lifelong condition to a curable disease. Effective chemotherapy of TB began in the 1940s, with the discovery and isolation of streptomycin (SM) and para-amino salicylic acid (PAS) around 1943. Before the use of pharmacotherapy patients would stay in sanatoria, where they were isolated from the general population, and treatment was based on getting rest with controlled exercise, fresh air and a healthy diet (38). Drug therapy resulted in a dramatic improvement in treated patients, but drug resistance quickly followed, leading to relapse in some patients. The search for new drugs then led to the discovery of the therapeutic benefit of isoniazid (INH) in 1953, pyrazinamide (PZA) in 1954, cycloserine (CS) in 1955, ethionamide (ETM) in 1956, rifampicin (RIF) in 1957 (first used clinically in 1966) and ethambutol (EMB) in 1962(26, 39). Looking at the history of medicine in general, and that of TB, the success in curing TB was a major milestone in the history of humankind, as the disease was always a leading cause of death.

The currently recommended first-line treatment regimen for new cases of drug sensitive TB comprises of INH, RIF, EMB and PZA (2). The four drugs are taken for 2 months in the intensive phase, followed by a 4-month continuation phase in which only INH and RIF are taken. In re-treatment cases, streptomycin is added to the intensive phase. Most TB cases respond favourably to this treatment regime. However, drug resistance, which has been associated with factors such as monotherapy and low drug exposure in patients, is a growing threat to the effective treatment of the disease.

The current first-line regimen was shown to be effective about 40 years ago (40) and remains highly effective and well tolerated. Treatment success rates with the first-line therapy usually exceed 85%, reaching as high as 98% in clinical trial settings (3), and the WHO estimated that 50 million deaths were averted due to TB treatment between 2000 and 2017 (1). However, the long duration of treatment may have a negative impact on compliance and increases the chances of adverse effects such as adverse drug reactions and drug interactions, which compromises the effectiveness of therapy.

In addition to the long duration, TB treatment is complicated by the need to use multiple drugs. Use of a cocktail of drugs is known to mitigate the development of drug resistance and improve the efficacy of therapy. Studies have shown "…rapid onset of isoniazid resistance among patients receiving monotherapy and the suppression of resistance when isoniazid was given in combination…" (26), leading to the use of multi-drug regimens. Drug resistance was known to occur as early as the 1950s (41), especially when monotherapy was used. More recently, it has also been found to be associated with low drug concentrations in patients on combination therapy.

In vitro studies suggest that low drug exposure leads to the development of drug resistance (42). Clinical studies have also shown correlation between low drug concentrations in patients and treatment failure (8, 43-45). These low drug concentrations may be due to pharmacokinetic factors such as malabsorption and pharmacogenetic variability in metabolism and excretion of the drugs, as well as factors like drug interactions and poor adherence to therapy. While resistance goes up, the discovery of new effective drugs is

declining and becoming more expensive, hence preserving existing therapies is of paramount importance.

Besides chemotherapy, surgical treatment has been used, more so before the advent of chemotherapy (23), and can still be used in cases resistant to drugs (46). Because drug treatment is much safer and more effective, it remains the treatment of choice. First-line drugs are used in drug sensitive cases while second line and other drugs are reserved for drug resistant ones.

2.6.1. First-line TB drugs

Isoniazid (isonicotinic acid hydrazide)

First synthesized in 1912, it was only tested for the treatment of tuberculosis in 1951 and by 1952 its therapeutic benefits had been observed (23). It is a pro-drug whose conversion to the active form is catalysed by catalase/ peroxidase, a bacterial enzyme encoded by the gene *katG*. The active metabolite inhibits the formation of mycolic acids, important components of the mycobacterial cell wall, through the inhibition of the NADH-dependent enzyme enoyl acyl carrier protein reductase, which is encoded by the gene *inhA*. Mutations in the genes *katG* and *inhA* result in INH resistance (33). Highly active against actively growing mycobacteria, INH plays a leading role in the treatment of TB but is inactive against dormant bacilli. It is inexpensive, potent (MIC is 0.1 – 0.7 μM), well tolerated and relatively safe and is regarded as one of the most important TB drugs (47).

Isoniazid can be administered orally, intravenously or intramuscularly. Bioavailability approaches 100% but is significantly reduced by food intake and peak plasma concentrations are attained in 0.5 – 2 hours (47, 48). It has been suggested that the drug forms condensation products with some carbohydrates that are not absorbable. N-acetyl isoniazid (AcINH) is the major metabolite of isoniazid, formed by the acetylation of isoniazid by N-acetyl transferase 2 (NAT2), mainly in the liver (49). It does not seem to exert any pharmacological effect and its kinetics have not been extensively studied. However, the rate of its formation in humans follows a bimodal distribution, which is genetically determined. Humans are classified as either fast or slow acetylators, and the ratio of the metabolite to analyte concentrations can be used to estimate the acetylator phenotype (20). The figure below shows the metabolism of INH to AcINH.

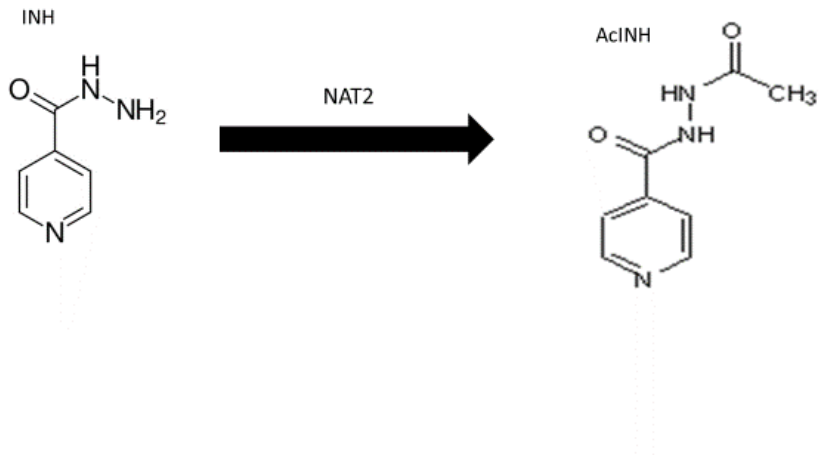

Figure 2.2: Acetylation of INH to AcINH

INH is metabolized to AcINH in a phase 2 acetylation reaction. The slow acetylator phenotype is at a higher risk of experiencing hepatotoxicity, which is the most serious adverse reaction of INH. Peripheral neuropathy may occur, especially in under-nourished individuals (48). Other metabolites include isonicotinic acid, isonicotinamide, monoacetylhydrazine and diacetylhydrazine. (47). Of all the metabolites, only monoacetylhydrazine is pharmacologically active and is said to be tuberculostatic and hepatotoxic (48). The drug is distributed throughout the body and together with its metabolites is mostly excreted via the kidneys (47). The drug is a white crystalline powder, soluble in water (125mg/ml) (48), weakly basic and stable at room temperature (47).

Pyrazinamide (analogue of nicotinamide)

Introduced in the 1950s PZA has been shown to have sterilizing effects in TB lesions. It is a pro-drug which is hydrolysed to the active pyrazinoic acid (POA) which is further oxidised to 5-hydroxypyrazinoic acid by xanthine oxidase. In acidic environments, POA is predominantly non-ionised and can passively permeate the mycobacterial cell wall. The inability of MTB to actively expel the drug makes PZA particularly effective. Two mechanisms of action have been suggested; the depletion of coenzyme A, and inhibition of phenolthiocerol dimycocerosate (PDIM) synthesis (33). PDIM are lipid components of the membrane which are involved in virulence of MTB (29). Unlike INH and EMB, PZA is more effective against persistors and less effective against actively growing mycobacteria (33).

PZA has good PK properties, with peak plasma concentrations attained in 1 – 2 hours and a half-life of 8 – 11 hours (41). Among the 1st line anti-tuberculosis drugs, it seems to have the

least variability in PK parameters, particularly in its absorption (41, 50), therefore factors like age and HIV status do not seem to affect plasma levels of this drug greatly. However, a few studies have shown an age-dependant variability of PZA pharmacokinetics in younger children (41, 51). The drug also has relatively good central nervous system penetration (51) although its efficacy in the relatively neutral CNS environment is unclear. PZA has been identified as a major determinant of treatment success, with low plasma concentrations being significantly associated with treatment failure (8). The major side effect is hepatotoxicity, but with currently used dosages this has not been frequently reported even though the drug is often administered with other potentially hepatotoxic drugs (41).

Ethambutol

Ethambutol (EMB) was first shown to have antimycobacterial effects in mice infected with drug resistant strains in 1961 and subsequently showed clinical benefit in combination with streptomycin and INH (23). It is also active against members of the mycobacterium avium complex (MAC) (52). Administered at a dose of 15-25 mg/kg/day, absorption is not significantly reduced by food intake (53) and peak plasma concentrations are reached in 2 to 4 hours. Plasma concentrations range from 0.95-7.5 μg/mL (39). The half life is short, and the drug is undetectable in plasma 24 hours after the last dose. With MICs ranging between 0.5–2 μg/ml (54), it is bacteriostatic against actively growing bacilli by blocking arabinosyl transferases, inhibiting the synthesis of arabinogalactan, an important polysaccharide cell wall component (55). Clinical studies show good penetration in lung, liver and kidney tissue (12). Its major adverse effect is that of visual disturbances, including decreased visual acuity, colour blindness and scotoma (blind spots), which may be due to optic or retrobulbar neuritis

(inflammation of optic or retrobulbar nerves) (56). This may occur on one or on both eyes and is usually reversible upon discontinuation of the drug. Less commonly, hypersensitivity reactions, gastrointestinal disturbances (nausea, vomiting, loss of appetite, abdominal discomfort), central nervous system (CNS) effects (dizziness, confusion and hallucinations), and peripheral neuropathy occur. Other effects, which may possibly be due to concurrent medicines have also been reported. Safety in children under 13 years old has not been established and information regarding use in pregnancy is inadequate. However, animal studies showed teratogenic effects at high doses in pregnancy (55).

The drug has a high polarity and as such is very water-soluble. It is a white crystalline powder, marketed as the dihydrochloride salt. It is also stable to heat and light (39). Due to its lack of a chromophore, EMB cannot be reliably detected by UV/Vis spectrophotometry.

Rifampicin

Rifampicin (RIF) is a semisynthetic rifamycin derived from the bacterium *Streptomyces mediterranei* (57). It binds to the β-subunit of bacterial DNA-dependent RNA polymerase, thereby inhibiting the transcription of messenger RNA from DNA. It is active against both actively growing and slowly metabolizing mycobacteria. Experiments with rifamycins started in 1957 and favourable results were reported in 1959 (23). Because it is well tolerated and highly effective, it has become one of the most important TB drugs. After the introduction of RIF, TB treatment became so successful that the search for new drugs slowed down and it took about 50 years for new TB drugs to be approved. However, its poor bioavailability from some formulations, particularly fixed-dose combinations (FDCs) and interaction with INH is

a cause for concern. It has been suggested that INH accelerates the degradation of RIF into the insoluble derivative 3-formyl rifamycin SV (3-FRSV) in the acidic stomach environment (58), thus reducing the bioavailability of RIF. Bioavailability is also reduced by concurrent food intake (49).

The major metabolic pathway is de-acetylation to 25-desacetyl-rifampicin (desRIF), a pharmacologically active metabolite. It is unknown whether this metabolite contributes to toxicity of the drug, but it is responsible for about 80% of antimicrobial effects of RIF in bile (49). The metabolism of RIF to desRIf is represented in Figure 2.3.

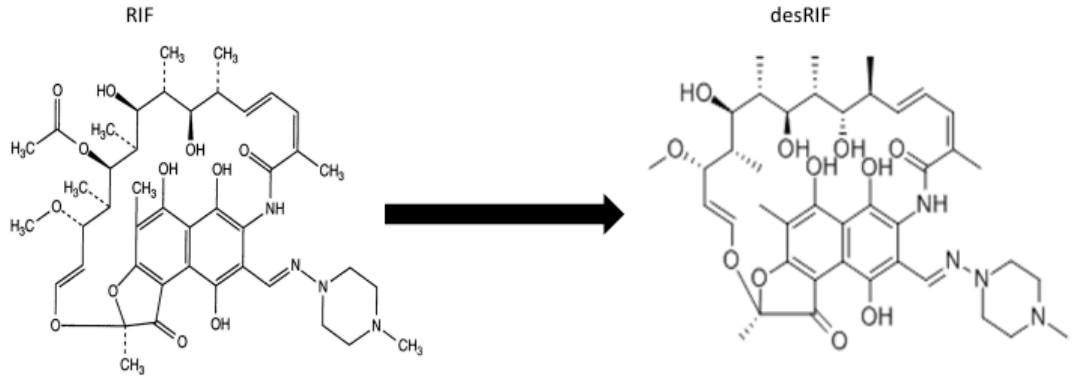

Figure 2.3: De-acetylation of RIF to desRIF

Rifampicin is a known hepatic enzyme inducer; it induces its own metabolism and that of other drugs, hence is susceptible to drug-drug interactions. Auto-induction of its metabolism results in a decrease in the maximum plasma concentration (Cmax) during long-term

administration. It is widely distributed in body tissues and fluids and is highly protein bound, mainly to γ-globulin (49). Rifampicin is generally well tolerated but may cause hepatotoxicity in about 1-2% of patients on monotherapy (59). Given the susceptibility of the bioavailability of RIF to external influences such as the presence of food and other drugs, and the changing metabolic rate, RIF probably has the highest variability in pharmacokinetics among the anti-TB drugs.

Streptomycin

Streptomycin, an aminoglycoside antibiotic, was the first drug to be successfully used in the treatment of TB. It was isolated from the soil bacterium *Streptomyces griseus* (33). By binding to the 16S subunit of rRNA it inhibits protein synthesis, thus exerting its bactericidal effects. Like other aminoglycosides, it is highly polar, a characteristic that gives it poor oral bioavailability, therefore is only administered parenterally. Intravenous, intramuscular and subcutaneous administration yield similar plasma concentration 2 hours post-dose (49). The drug may be considered as a first-line drug but is usually reserved for re-treatment cases of drug-susceptible TB.

2.6.2. Second and third-line TB drugs (MDR-TB treatment)

Fluoroquinolones

This is a class of drugs including such drugs as ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin, which act by inhibiting the mycobacterial enzyme DNA gyrase (a type II topoisomerase) (33). Although fluoroquinolone-based therapy resulted in earlier sputum conversion in clinical studies, these regimens resulted in higher rates of relapse compared to the standard 1st line regimen (2, 60), hence this class continues to be used as part of 2nd line therapy. Moxifloxacin has been most widely used among the fluoroquinolones for TB treatment, but levofloxacin is thought to be less likely to cause QT prolongation, the major adverse effect of this class of drugs (61). Other adverse effects include gastrointestinal disturbances, rashes, dizziness and headache (54).

Capreomycin, amikacin and kanamycin

Capreomycin comprises of 4 isomers (lA, IB, IIA, lIB) which are cyclic peptide antibiotics, the majority of which are IA and IB in pharmaceutical formulations (33). Kanamycin is an aminoglycoside antibiotic, amikacin is its semi-synthetic derivative. Both capreomycin and the aminoglycosides inhibit protein synthesis (54). Aminoglycosides act by binding to the 30S ribosomal subunit of MTB (33) while capreomycin binds to the 70S ribosome, inhibiting mRNA- tRNA translocation (54). Second-line TB therapy must include any one of these injectable drugs and the choice of which one to use is based on the likelihood of efficacy and availability (62). The most common adverse effects of both antibiotic families are ototoxicity and nephrotoxicity (54).

Ethionamide

Ethionamide (2-ethylisonicotinamide), a derivative of isonicotinic acid, was introduced as an anti-mycobacterial drug in 1956 (54). It is a pro-drug, like INH, which, when activated inhibits the same molecular target as INH (*inhA*), thus inhibiting mycolic acid synthesis. Mutation in *inhA* results in resistance to both drugs (63). Frequent side effects include abdominal discomfort, nausea, vomiting, anorexia and hypothyroidism when used concurrently with para-aminosalicylic acid (PAS) (54).

Cycloserine

Cycloserine is a 2nd line anti-TB drug used in the treatment of MDR TB. It was discovered in 1954 as a secretion of *Streptomyces orchidaceus* and its clinical benefit was published a year later (64). This analogue of the amino acid D-alanine, a key component of peptidoglycan, inhibits peptidoglycan synthesis thus preventing proper cell wall formation (33). It is rapidly degraded under acidic to neutral conditions and stable in alkaline solutions (49). About 10-50% of treated patients experience neuropsychiatric adverse effects, such as psychosis and seizures, which are probably dose-dependent (64). Because of the neuropsychiatric effects, it is likely to have high central nervous system (CNS) permeability and hence be useful in treating TB meningitis.

Para-amino salicylic acid

One of the first anti TB drugs to be discovered, PAS is still effective. It is thought to inhibit dihydropteroate synthase, an enzyme involved in folate biosynthesis (33). It is a bacteriostatic agent which is readily absorbed from the gastrointestinal tract (GIT) (49). Because of possible gastrointestinal irritation, concurrent food intake is recommended. With the introduction of more efficacious, less toxic drugs, the use of PAS is now mainly limited to treating XDR-TB.

2.6.3. New and repurposed drugs

New drugs were approved for the treatment of TB after about 50 years of no new discoveries. Previously registered drugs for other indications have also been re-purposed for MDR-TB.

Bedaquiline

Bedaquiline, a diarylquinoline that is currently in Phase III clinical trials, received conditional approval by the Food and Drug Administration (FDA) in December 2012. It has been included in the WHO's guidance for the treatment of XDR TB since no cross resistance with other drugs has been reported (46, 65). It blocks mycobacterial ATP synthase, a novel

mechanism of action, and is bactericidal to both replicating and non-replicating bacteria (65). Early bactericidal activity (EBA) is comparable to INH and RIF after 5 days and is active against drug sensitive and drug resistant TB (including XDR) TB (33). Although it causes QT prolongation, no clinically significant adverse outcomes relating to QT prolongation have been reported. Elevation of liver enzymes has been reported but no serious hepatotoxicity has been reported (66).

Delamanid

Delamanid belongs to the nitro-dihydroimidazoxazole class of drugs, a derivative of the antimicrobial metronidazole, which was approved for use by the European Medicines Agency (EMA) and Japan's Pharmaceuticals Medical Devices Agency in 2014. It is a pro-drug which is converted to the active desnitroimidazole and probably inhibits methoxy-mycolic and keto-mycolic acid synthesis (33, 66). Effective in replicating and non-replicating organisms, it has good tolerability (67) and EBA and potency, with an MIC of 0.006–0.024 mg/ml. No cross resistance with first-line drugs has been found.

Carbapenems

This is a class of broad spectrum anti-microbials, active against gram positive and negative, aerobic and anaerobic bacteria. Pre-clinical studies in mice and some case reports have shown efficacy of the drugs in TB treatment (66). Meropenem, imipenem and ertapenem have been

used to treat MDR and XDR TB with some success (60). However, use is limited by the need for parenteral, mainly intravenous administration.

Linezolid

Linezolid is an oxazolidinone antimicrobial used for XDR TB. It has a high frequency of side effects mainly of hematological and neurological nature (46, 66). In a clinical trial "…82% of patients developed significant adverse events, namely myelosuppression, peripheral neuropathy and optic neuropathy." (60). It acts by binding to the ribosomal 50S subunit, thus interfering with protein synthesis (33). With a narrow therapeutic index, TDM and MIC measurements are recommended (68). Because it has remarkable sterilizing effect and an unfavourable toxicity profile, new oxazolidinones including sutezolid (PNU-100480), posizolid (AZD-5847), torezolid, and radezolid are under development.

Clofazimine

Developed as an anti-leprosy agent in the 1950s it was overshadowed as a TB drug by more effective drugs such as INH introduced in the same period (46). The drug has a long duration of action and is effective against dormant bacilli. It rapidly attains high concentrations in macrophages making it highly desirable in treating TB (66). Pre-clinical studies have shown good activity against drug resistant strains and low toxicity (66). It has been suggested that clofazimine may shorten treatment of MDR TB. Its major toxicities include skin pigmentation (and causes urine discoloration) and GI upset.

Pretomanid

A bicyclic nitroimidazofuran compound currently in phase III clinical trials, it releases nitrogen oxide (NO) which inhibits ATP synthesis in non-replicating mycobacteria (69). Pretomanid also inhibits ketomycolates that are necessary for cell wall synthesis. It is active against MDR TB and no cross resistance with other TB drugs has been seen. It is also active against non-replicating bacteria and is less effective when PZA is not present (33).

2.7. Bioanalysis

Bioanalysis refers to the qualitative or quantitative determination of analytes in biological fluids such as blood, plasma and urine. Analytes may be endogenous (e.g. proteins, peptides, nucleic acids) or exogenous (drugs, metabolites, environmental pollutants). It is frequently applied throughout the drug development process, from pre-clinical, clinical, to pharmacovigilance studies. Bioanalysis is a multi-step process, starting from sample collection, through sample preparation, analysis and detection to data analysis and reporting. An analytical method provides step by step details of the techniques, materials and equipment used in performing an assay. The method development process tries to identify parameters that are critical in a bioanalytical method, and to minimise any factors that may be detrimental to the sensitivity, accuracy and robustness of the assay. The choice of analytical methodology takes into account various factors, most importantly the physicochemical properties of analytes, the purpose of the analysis and availability of resources (70).

Commonly used techniques for the quantitative determination of drugs and metabolites in biological matrices include gas chromatography (GC), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) for separation of analytes, coupled to detectors such as spectrophotometers (ultraviolet/visible (UV/vis), infrared (IR) and fluorescence), mass spectrometry (MS) and electrochemical methods. High performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is probably the most widely used bioanalytical technique (71).

2.7.1. Mass spectrometry

Before much method development takes place, it is necessary to decide on the detection technique suitable for the analytes. For MS analysis, analytes must first be infused into the instrument, usually as neat solutions, using an infusion pump. Infusion flow rates typically range from 1 to 20 µl/min. The mass spectrometer will detect the mass to charge ratio (m/z) of the molecular ion and that of any fragment(s) produced when the analyte passes through the collision cell. In many cases molecules will have only a single charge, rendering the m/z equivalent to the molecular mass of the species. The specific transition from a precursor molecule (parent/precursor ion) m/z to fragment or product (daughter/product ion) m/z is highly specific and forms the basis of detection. Infusion of a known reference standard produces a unique transition pattern (from parent ion to fragment ion(s)) which will be used to identify the presence of that compound when samples are injected into the MS. When the mass spectrometer is set-up to monitor the fragmentation of an analyte into more than one product ion it is referred to as multiple reaction monitoring (MRM).

An example of the MRM for INH is shown in Fig 2.4 below.

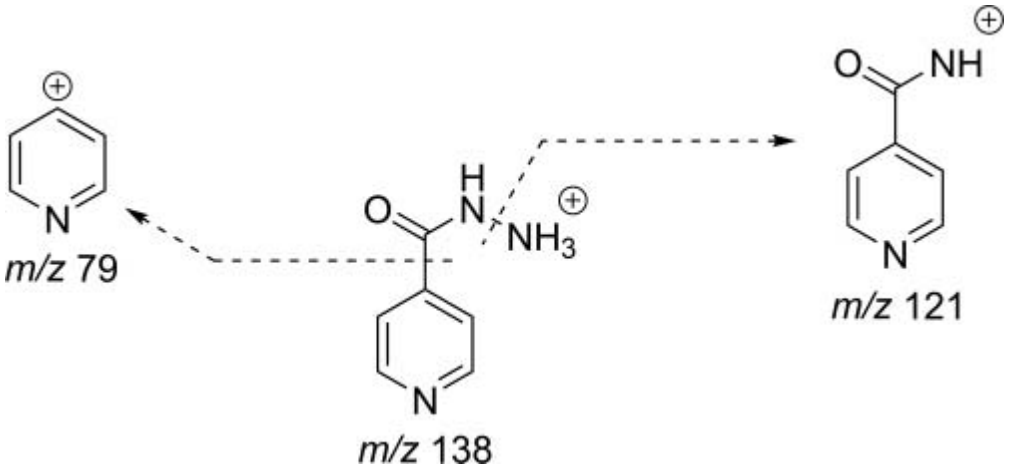

Figure 2.4: Fragmentation of isoniazid in mass spectrometry

The mass spectrometer comprises of three basic components namely: ion source, mass analyser and detector (72). The ion source operates at atmospheric pressure while the mass analyser and detector are under vacuum. Ions are driven from the ion source to the detector through the mass analyser by both a pressure gradient and potential differences between the compartments (72).

Mass spectrometry analyses charged molecules in the gas phase. Most samples to be analysed, however, are liquid solutions. The ion source enables the transition of molecules from liquid solution to ionized gaseous molecules (72). Generally polar solvents, like water, methanol, acetonitrile, are used as mobile phases as they aid in the ionisation process (73). Volatile mobile phases are preferred for easier evaporation in the ion source. The two most commonly used source types are Electrospray Ionisation (ESI) and Atmospheric Pressure Chemical Ionisation (APCI).

The mass analyser sorts and separates ions according to their mass to charge ratio (m/z value) (72). Types of mass analysers include magnetic or electric sector mass analyser, linear quadrupole ion trap (LIT), three-dimensional quadrupole ion trap (QIT), orbitrap and time-of-flight mass analyser (TOF).

From the mass analyser the separated ions are transferred to the detector which measures their concentration. The ions and their concentrations are displayed on a graph called a mass spectrum. The peak height is proportional to the number of ions that hit the detector (72).

2.7.1.1. Matrix Effects

The major drawback of mass spectrometry is the "matrix effects" phenomenon. This occurs when other sample components interfere with the ionisation of the analyte(s) and/or droplet formation and evaporation. It affects aspects of method performance including sensitivity, reproducibility and accuracy (74). Matrix effects, when present, may be reduced or avoided by altering the sample clean-up process, chromatography or using stable isotopically labelled (SIL) internal standards (75).

2.7.1.2. Internal standards

Internal standards are used to compensate for process and instrument related variability, based on the assumption that the internal standard will be subjected to the same processes,

matrix effects and instrument conditions as the analyte. They are usually structural analogues of analytes or the analytes which have some of the atoms substituted by stable isotopes, commonly called "stable isotope labelled (SIL) internal standards". SIL internal standards resemble the analytes more than structural analogues and are therefore preferred. Most quantification processes use the analyte to internal standard ratio rather than the absolute analyte signal due to the vulnerability of analyte signal to matrix effects (74). Generally, ^{13}C, ^{15}N or ^{18}O-labeled internal standards are preferable to deuterium labelled analogues because deuterium labelled IS may exhibit slightly different physicochemical properties relative to the analytes and back exchange reactions can occur between deuterium labelled internal standards and analytes (76). It is ideal to use one internal standard for each analyte when there is more than one analyte.

2.7.2. Liquid chromatography

Chromatography involves an intricate physicochemical interaction between analytes and the stationary and mobile phases. Each chemical entity has a distinct affinity for the stationary phase relative to the mobile phase, hence chemical entities will have different velocities and separate as they flow through the stationary phase column (77). In its various forms it is probably the most widely used analytical technique for separating compounds in a sample mixture (78). Depending on the physical state of the mobile phase, chromatography may be classified as gas chromatography (GC), supercritical fluid chromatography (SFC) and liquid chromatography (LC). Liquid chromatography is further divided into sub-types such as reverse phase (RP), normal phase, ion exchange and hydrophilic interaction liquid chromatography (HILIC). In reverse phase liquid chromatography, analytes are separated based on their relative hydrophobicity; the more hydrophobic an analyte is, the longer it is

retained on the stationary phase. Elution of compounds can be achieved through either isocratic or gradient flow. Isocratic flow is when a constant proportion of organic versus aqueous mobile phase flows throughout the run whereas in gradient flow the proportion of organic relative to aqueous mobile phase changes according to pre-selected program. Gradient HPLC gives better sensitivity to analytes with longer retention times since it results in narrower and higher peaks.

2.7.3. Sample preparation

Pre-treatment of biological samples is necessary to make the sample more suitable for analysis and prevent deleterious effects on the LC-MS system. It is sometimes called sample clean-up and aids in ensuring maximum sensitivity and reproducibility (79). This is usually the most tedious and time-consuming step in bioanalysis. It is performed to remove sample components that would potentially interfere with detection of the analyte or cause malfunction of the analytical instrument. The most commonly used sample preparation methods include protein precipitation (PP), liquid-liquid extraction (LLE) and solid phase extraction (SPE).

Protein precipitation

PP is a commonly used sample processing technique which involves the use of salts, acids, heat or organic solvents to precipitate proteins in the sample. It is simple and can be used for both polar and non-polar molecules. The precipitated proteins form a pellet at the bottom of the tube when the sample is centrifuged, and the supernatant can be directly injected into the

LC-MS/MS system or subjected to further sample clean-up. PP is recommended whenever high-throughput and low extraction variability are required (80)

Liquid-liquid extraction

LLE is highly selective and has been shown to give extracts with the least ion suppression (79). An aqueous sample is mixed with an immiscible organic solvent and the analyte partitions into the organic phase. It may, however, not be suitable for hydrophilic compounds and the formation of emulsions can make it difficult to isolate the extraction. Its main disadvantages are the need for large amounts of organic solvents and the fact that it is not possible to automate LLE when the solvent needs to be evaporated (73).

Solid phase extraction

SPE involves the use of silica or polymer-based sorbents to adsorb analyte(s), washing and eluting with suitable solvents. It can be easily automated, and a wide variety of sorbents are available for wide ranging selectivity, hence it is the most popular sample preparation technique (79).

2.8. Bioanalysis of first-line TB drugs

First-line TB drugs have been in clinical use for over 50 years, yet their PK and PK-PD relationships are not fully understood. Several studies have shown that these drugs are frequently below the expected concentrations in patients given the recommended doses (7-9, 50, 81, 82). This necessitates therapeutic drug monitoring (TDM) to ensure patients attain therapeutic drug concentrations with minimal toxicity.

Assays have been developed for the quantification of these drugs individually and in combination. Quantification of all drugs is ideal because it saves both time and resources. This was first done by Song *et al.* who developed a method for quantifying the four first- line drugs and the metabolites AcINH and desRIF (20). In this method the authors used structurally related compounds as internal standards, namely rifabutin for RIF and desRIF, and 6-aminonicotinic acid for the rest of the analytes. Baietto *et al.* also developed a method for the simultaneous quantification of first-line anti-TB drugs (18). Similarly, the authors also used other compounds (thymidine and 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline)) as internal standards, hence matrix effects and stability concerns may not have been adequately compensated for. Prahl *et al.* developed a method for the analysis of all four drugs using deuterated internal standards for each analyte (19). This method, however, did not include metabolites. In this project, we developed and validated a method for the simultaneous quantification of the four first-line oral anti-TB drugs and the two major metabolites for application in TDM and clinical PK studies. This method furthermore incorporates the use of isotopically labelled structural analogues of each analyte, thereby allowing adequate compensation for any potential variability during sample preparation and on-instrument.

Chapter 3 : AIM, OBJECTIVES and RATIONALE

3.1. Aim

The aim of this project was to develop and subsequently validate an assay for the quantification of the four first-line oral anti-tuberculosis drugs and the metabolites acetyl isoniazid and des-rifampicin in human plasma.

3.2. Objectives

1. To develop a method that can quantitate isoniazid, rifampicin, ethambutol, pyrazinamide, acetylisoniazid and desacetylrifampicin simultaneously in human plasma.
2. To validate the method that can quantitate the four first-line anti-tuberculosis drugs and the two major metabolites.

3.3. Rationale

Tuberculosis is a leading cause of morbidity and mortality worldwide whose treatment can be highly successful but may be compromised by factors such as drug resistance and variability in the pharmacokinetics of the drugs. Many gaps still exist in the characterization of the pharmacokinetics, pharmacodynamics and the pharmacokinetics-pharmacodynamics

relationship of the first-line drugs, despite all of them having been introduced more than 50 years ago. Some metabolites of these drugs are also pharmacologically active and need to be monitored. Quantification of metabolites may give useful information on the pharmacology of these drugs. Studies are required to generate knowledge to cover these knowledge gaps. Simple, accurate and robust assays are necessary for quantifying these drugs and metabolites in biological fluids. It is ideal to measure all drugs in a single assay to allow processing of samples in a simple, timely and cost-effective manner. A method was therefore developed and validated for this purpose. This method can also be used to carry out therapeutic drug monitoring of all four drugs in TB treatment cases that are problematic, either due to poor response to treatment or to adverse drug reactions.

Chapter 4 : METHOD DEVELOPMENT

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a widely used technique in bioanalysis. One of its major advantages is the ability to analyse a wide range of compounds without complicated derivatisation steps. It is also highly sensitive and selective. Therefore, it was chosen for this project. Ethical approval for use of human plasma during method development and validation was granted by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (approval letter attached in Appendix).

Selecting a suitable LC system is usually one of the first steps in method development. It was planned that this project would be carried out using reverse-phase chromatography on a micro-LC system, which uses columns and tubing of minute internal diameters, less than 1 mm, and flow rates less than 100 µl/min. Micro-LC is not as commonly used as normal flow-LC, but is thought to have several advantages, including fast analysis time, use of less materials (mobile phase, sample size and stationary phase), more efficient chromatographic separation and good sensitivity (due to use of less mobile phase hence less dilution). The next section describes the method development that was carried out on an Eksigent micro-LC system, coupled to an AB Sciex API 2000 triple quadrupole mass spectrometer.

4.1. Micro-LC and API 2000

MS has been used successfully in previous analytical methods to detect all of the analytes in this project. Solutions of reference standards of the 6 analytes at 500 ng/ml were prepared from 1 mg/ml stock solutions and were individually infused. Table 4.1 shows the four most abundant product ions obtained for each analyte after fragmentation in the collision cell.

Table 4.1: MRM transitions for the 4 most abundant product ions

	m/z (Da)					
Analyte	AcINH	desRIF	EMB	INH	PZA	RIF
Precursor	180.1	781.1	204.2	138.1	124.1	823.2
Product 1	138.2	748.7	116.3	121.0	107.2	791.5
Product 2	120.5	748.0	115.5	119.3	104.0	790.3
Product 3	121.1	749.1	113.7	118.5	103.8	790.8
Product 4	137.5	746.9	114.4	117.4	102.1	791.6

Only the 2 most abundant product ions were chosen to be used for multiple reaction monitoring (MRM). The most abundant product ion would be used for quantification while the 2nd most abundant would be used as a qualifier, to confirm the identity of the compound.

The infusion process also produced data on the optimum potential differences between different compartments in the MS. These were: declustering potential (DP), focussing potential (FP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP). The results are shown in Table 4.2 below.

Table 4.2: MS parameters optimized during analyte infusion

Analyte	Instrument parameters (volts)						Dwell (msec)
	DP	FP	EP	CEP	CE	CXP	
AcINH	16	290	8	12	27	12	150
desRIF	6	370	5	36	41	56	150
EMB	16	290	9	12	29	10	150
INH	16	230	8.5	10	31	58	150
PZA	21	330	8.5	10	129	46	150
RIF	6	370	10	24	45	58	150

Initially the source parameters were arbitrarily chosen to fall within ranges commonly used.

Table 4.3 shows the source parameters used.

Table 4.3: Initial source parameters on micro-LC system

Parameter	CUR (psi)	TEM (^{0}C)	GS1 (psi)	GS2 (psi)	CAD (psi)	Ionspray voltage (V)
Value	10	250	20	20	10	5000

A polar C18 column (Phenomenex Lunar Omega) with 1.6 µm particles, 0.5 mm internal diameter and 5 cm length was chosen. It was speculated that since 4 out of the 6 analytes are highly polar, a more polar stationary phase would have better retention than a conventional RP C18 column. A short column (5 cm) was preferred as it reduces analysis time (shorter retention and equilibration time) and reduces system back pressure. A gradient was chosen over an isocratic elution because the polarity range among analytes is wide. Mobile phase A was a 0.1% formic acid (FA) aqueous solution and mobile phase B was 0.1% FA in a 1:1 mixture of acetonitrile and methanol. The following gradient (Table 4.4) was used at a flow rate of 50 µl/min:

Table 4.4: Initial gradient table on the micro-LC system

Time (min)	% A	% B	Flow rate (µl/ml)
0	90	10	50
0.5	90	10	50
1	10	90	50
3	10	90	50
3.1	90	10	50
5	90	10	50

At first sensitivity was relatively low, and PZA practically had no peaks. Chromatography was also poor, especially for INH and AcINH which had split peaks. The chromatograms in

Figure 4.1 and Figure 4.2 below were produced after injecting a solution containing all analytes at 10 µg/ml.

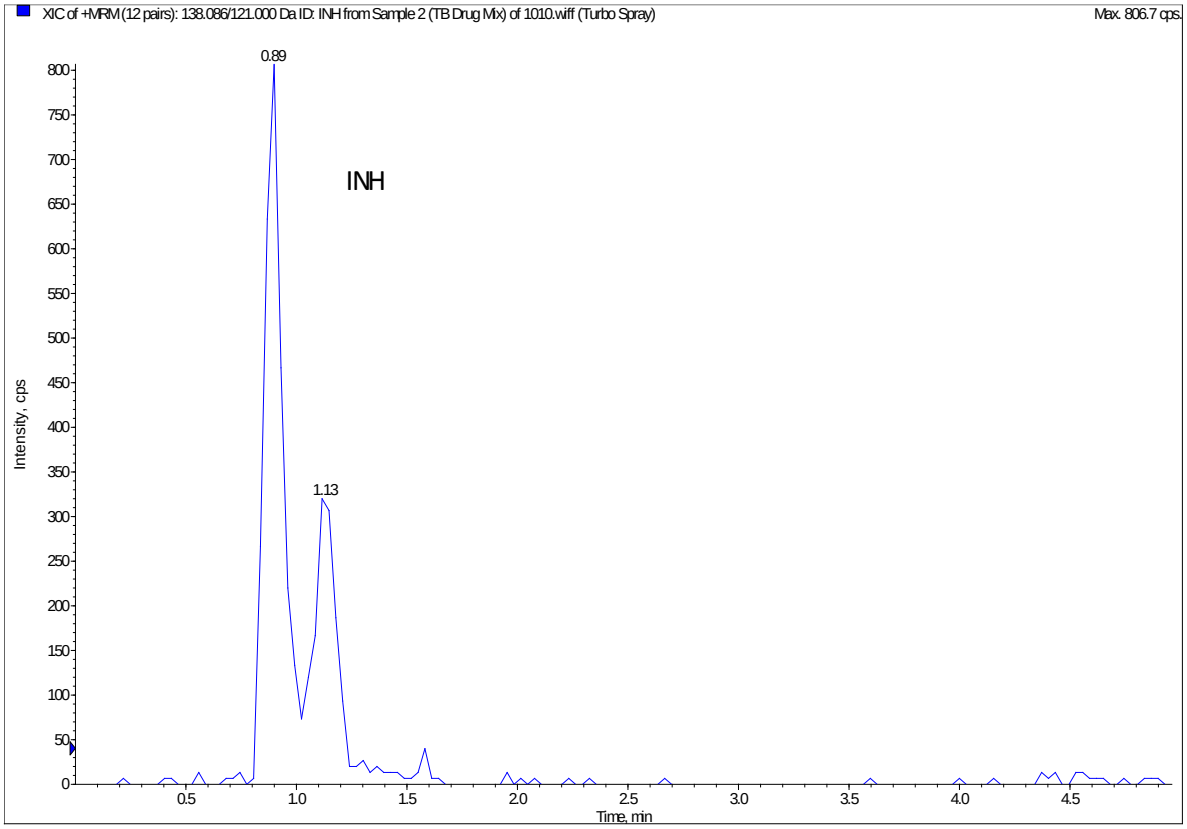

Figure 4.1: Chromatogram of INH in early method development

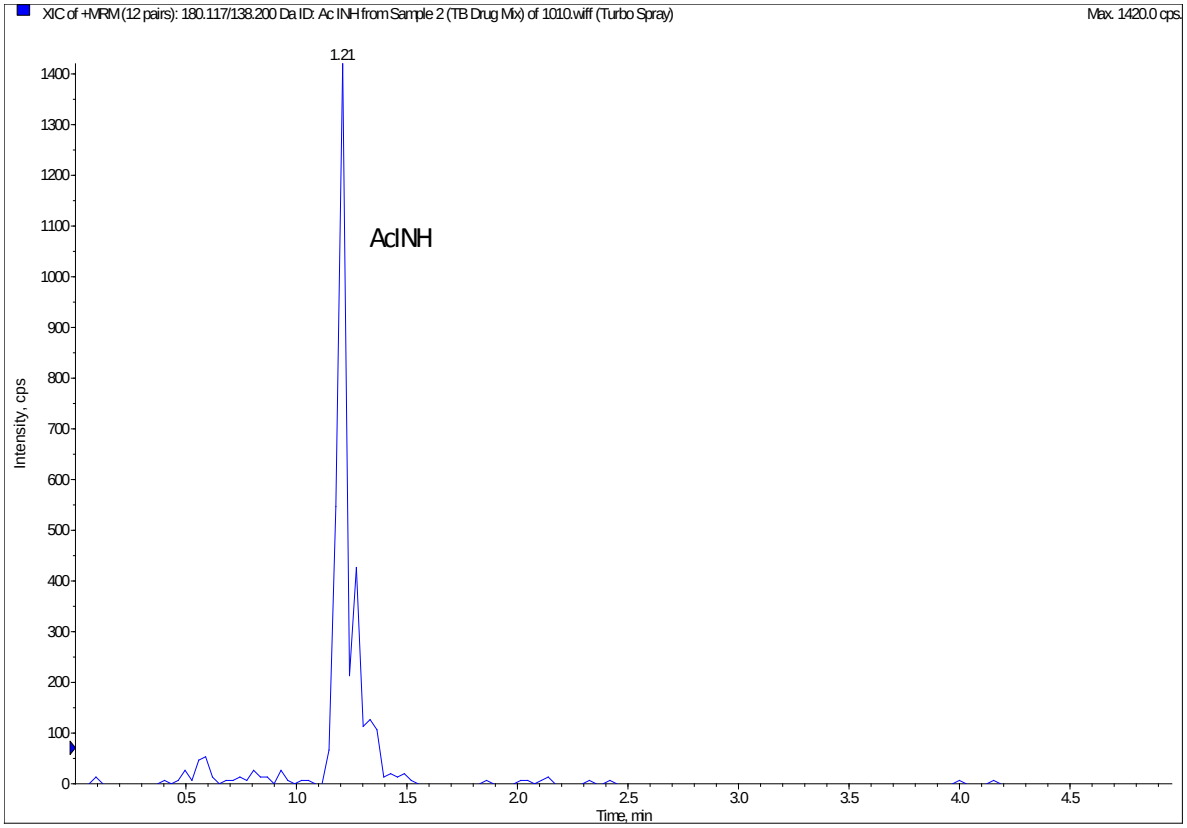

Figure 4.2:Chromatogram of AcINH in early method development

The mass spectrometer was cleaned and re-calibrated, and analytes were infused again. The same product ions were obtained as those obtained during the first infusion for all analytes except PZA. In the first infusion, only product ions greater than 100 amu (atomic mass units) were detected, therefore the most abundant fragment of PZA with m/z value of 78 was not detected, resulting in lack of sensitivity for this analyte. The following transitions (Table 4.5 and 4.6) were selected from the second infusion:

Table 4.5 MRM transitions after 2nd infusion

analyte	m/z					
	AcINH	desRIF	EMB	INH	PZA	RIF
precursor	180.1	781.3	205.2	138.1	124.0	823.4
product 1	137.7	749.2	116.1	120.9	78.9	791.4
product 2	121.1	399.2	115.4	118.6	107.2	151.1

Table 4.6: Re-infusion of internal standards on the micro-LC system

Internal standard	Q1 m/z	Product ion m/z
AcINH-d4	184	142
desRIF-d3	785	752
EMB-d4	209	120
INH-d4	142	83
PZA-15N,d3	128	84
RIF-d3	826	794

Injection of a solution with 1 µg/ml of each analyte produced the following chromatogram:

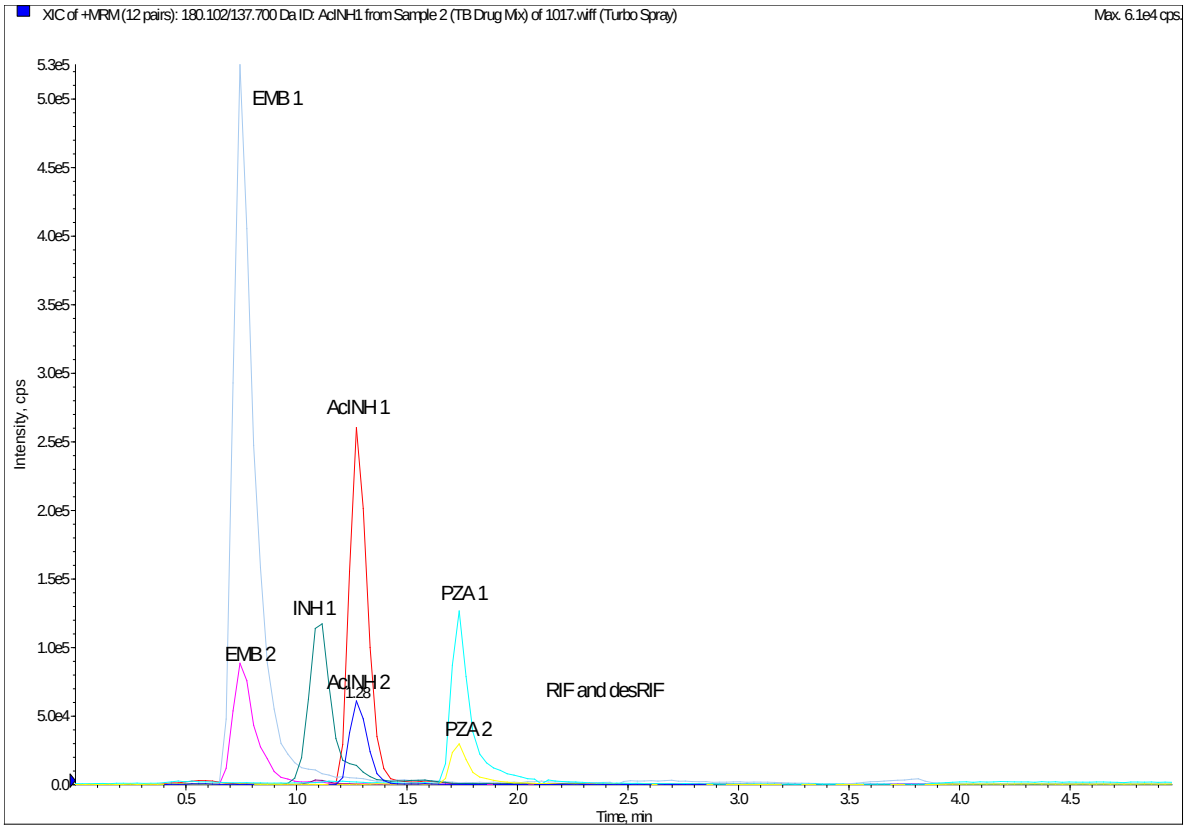

Figure 4.3: Chromatogram after re-infusion

The chromatogram above shows good peak retention and resolution. However, RIF and desRIF had low intensities.

4.1.1. Effect of ascorbic acid

RIF and its metabolite are thought to undergo oxidation, and this was suspected to be causing low signal intensities for these analytes (83). Ascorbic acid, an anti-oxidant, is likely to counteract the oxidation. Ascorbic acid was therefore added to injection solutions at 25, 50 and 100 µg/ml concentrations and the results are shown in Figure 4.4.

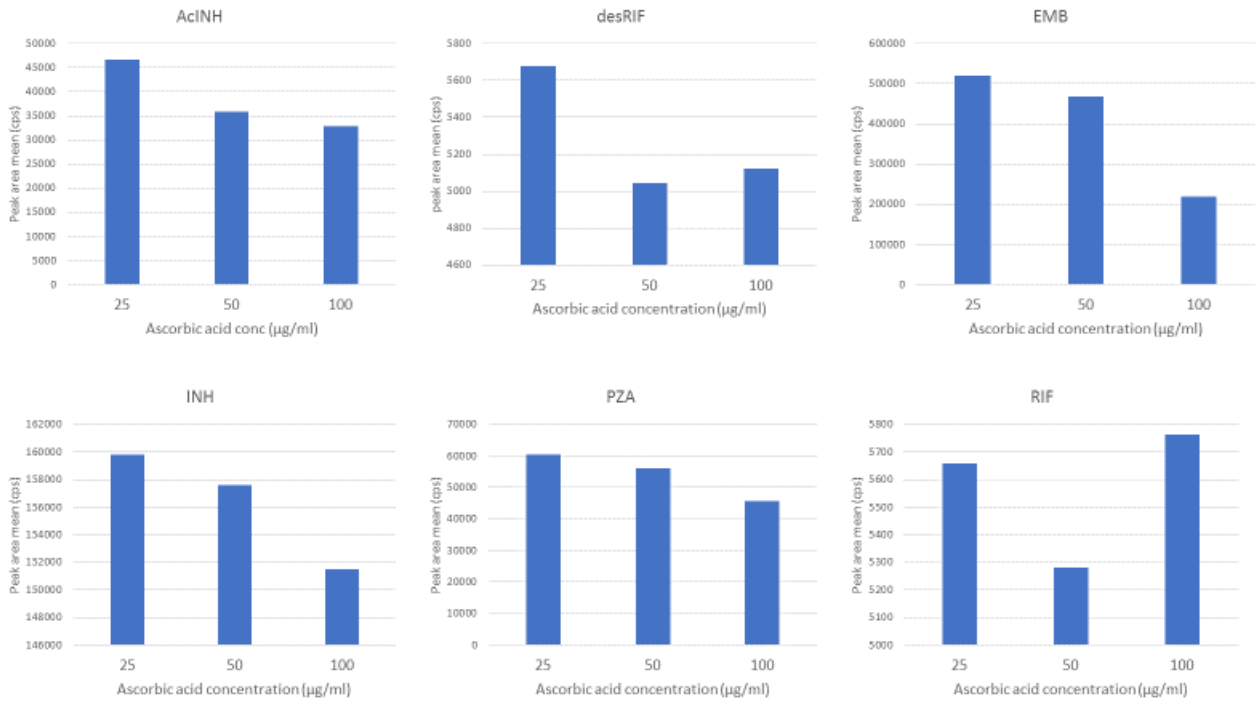

Figure 4.4: Effect of ascorbic acid

For all analytes except RIF and its metabolite, there was a clear downward trend in peak area as the ascorbic acid concentraton increased. Peak areas for RIF and desRIF were relatively high with 25 µg/ml ascorbic acid, dropped when 50 µg/ml was used but rose again when 100 µg/ml was used. Although peak areas were lower in the presence of ascorbic acid, peak shapes for INH, RIF and desRIF seemed to improve. Ascorbic acid may alter the pH of the mobile phase, thus affecting peak shapes. Poor peak shapes of INH in the absence of ascorbic acid were seen when fresh mobile phase was used but seemed to improve with older mobile phase. A possible chemical interaction between MeOH, a proton donor, and ACN, a proton acceptor, could result in alteration of the mobile phase character, hence the change in peak

shape. The figures (Figure 4.5 and 4.6) below show the differences in peak shape in solutions with and without ascorbic acid when fresh mobile phase was used.

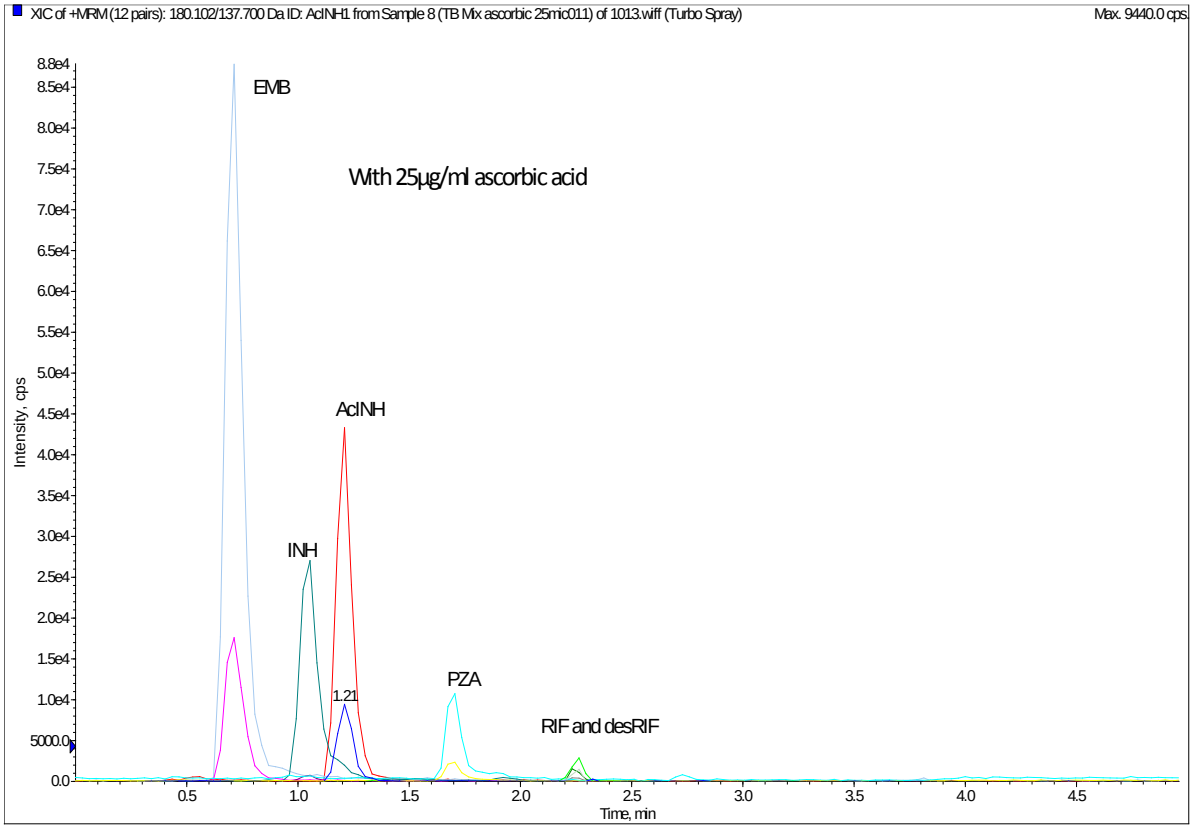

Figure 4.5: Chromatogram when injection solution contains ascorbic acid

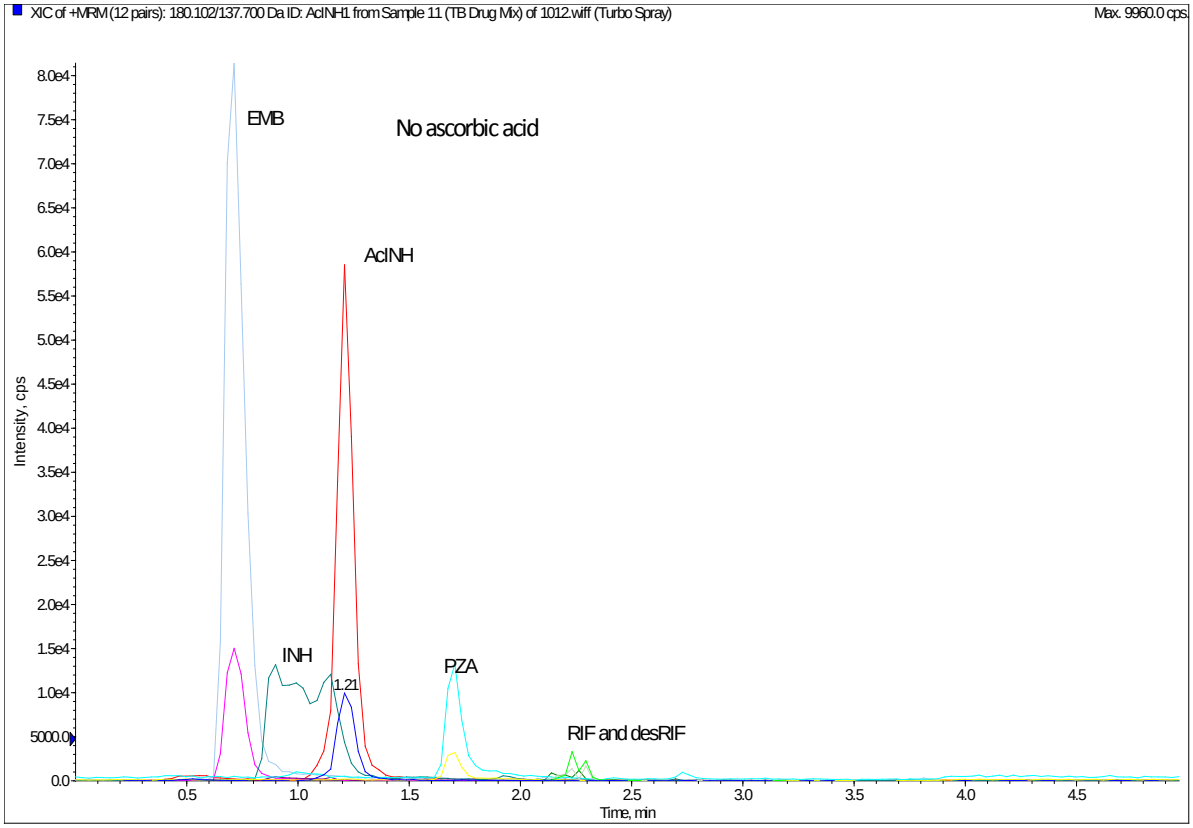

Figure 4.6: Chromatogram when injection solution does not contain ascorbic acid

4.1.2. Organic mobile phase composition

The impact of changing the organic mobile phase (Mobile phase B) composition was also tested using mixtures of different proportions of methanol: acetonitrile. All analytes except INH and PZA had the highest peak areas with a 50:50 mixture. The highest peak areas for INH and PZA were seen with a 40:60 (MeOH:ACN) mixture although the difference compared to 50:50 was very small. Peak areas for RIF and desRIF were not satisfactory with all mobile phase compositions. The results are summarized in Figure 4.7.

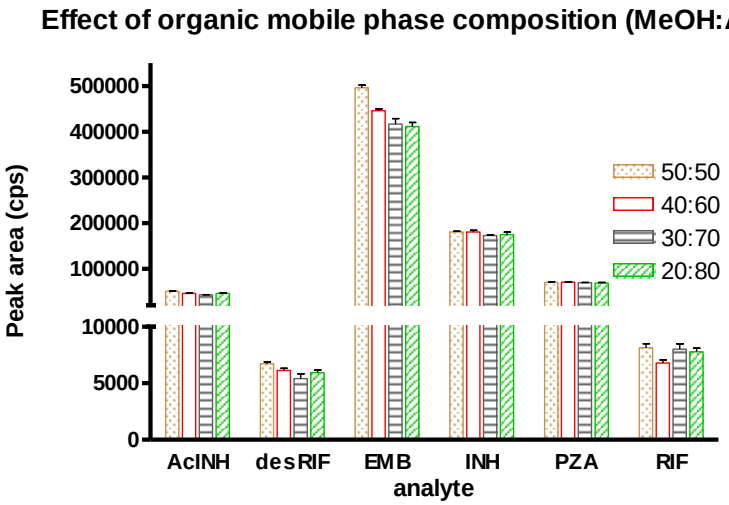

Figure 4.7: Effect of varying organic mobile phase composition

4.1.3. Source parameters

4.1.3.1. Temperature (TEM)

The effect of source temperature was tested, and the results are summarized in Figure 4.8.

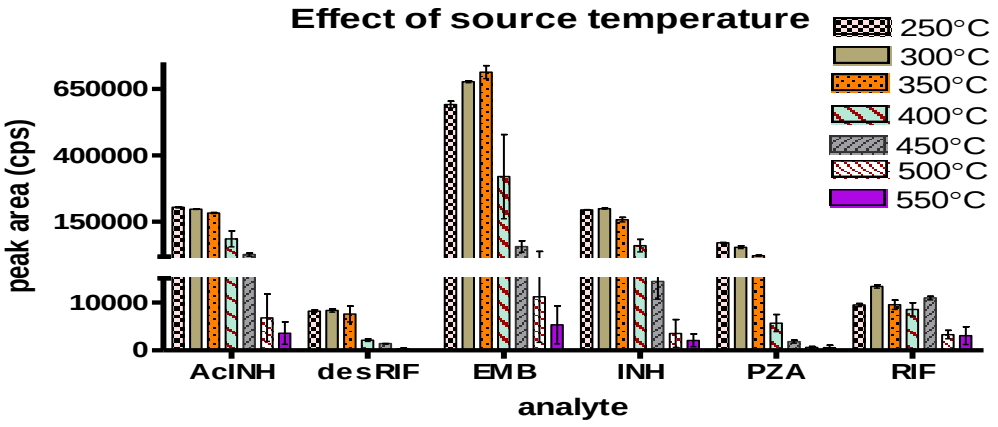

Figure 4.8: Effect of source temperature on peak intensity using micro-LC

Generally, sensitivity dropped as temperature increased for all analytes, except for RIF which did not show a clear trend. Both RIF and desRIF had low peak intensities at all temperature levels.

4.1.3.2. Ionspray voltage (IS)

Ionspray voltage was optimised and the results are shown in Figure 4.9 below:

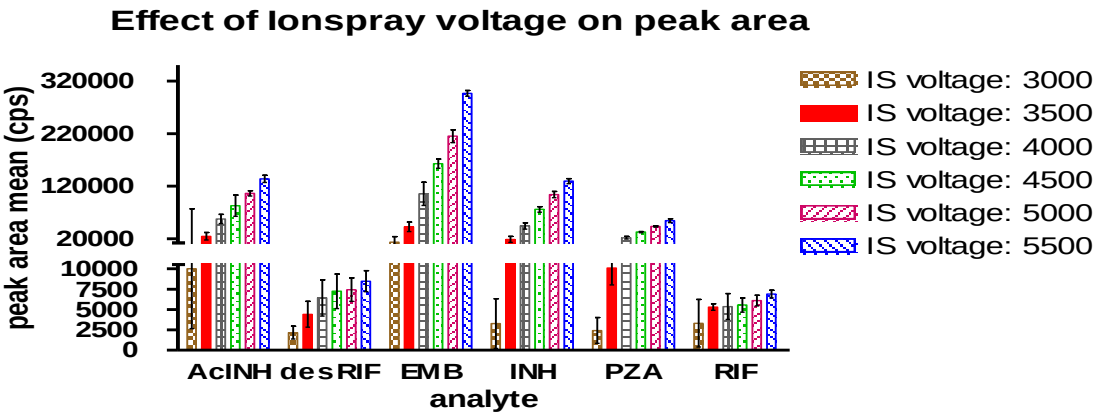

Figure 4.9: Effect of Ionspray voltage on peak areas

Peak areas increased as ionspray voltage increased for all analytes. Peak intensity was still not satisfactory for RIF and desRIF at all ionspray voltage levels.

4.1.3.3. Gas 1 and Gas 2

Gas 1 and Gas 2 were also optimized, and results are presented in Figures 4.10 and 4.11.

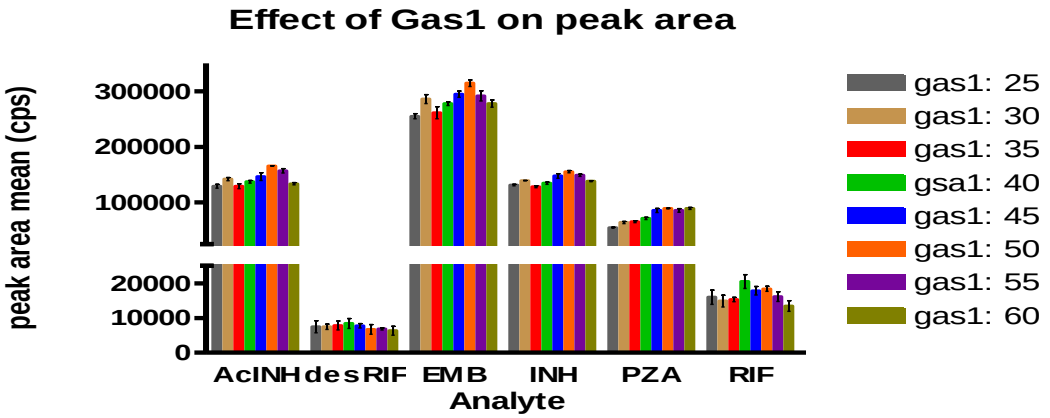

Figure 4.10: Effect of Gas 1 on peak areas

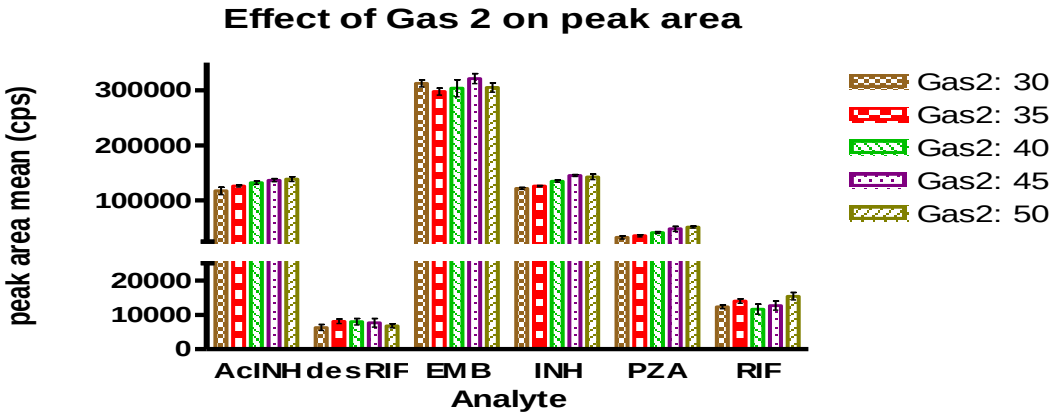

Figure 4.11: Effect of Gas 2 on peak areas

Optimizing source parameters did not significantly improve RIF and desRIF peak intensities. Reproducibility was also poor for these two analytes. Different gradients were tried, and stock solutions were sonicated before preparing injection solutions to ensure total dissolution of all analytes. These strategies also did not improve RIF and desRIF sensitivities. It was then decided that the project be moved to a more sensitive mass spectrometer: API 3200 Q-trap. The micro-LC system would continue to be used.

4.2. Micro-LC with API 3200 Q-trap

All analytes dissolved in 50% methanol were infused at 10µl/min using a syringe pump, the following transitions (Table 4.7) were obtained:

Table 4.7: Product ions from infusion on a 3200 Q-trap mass spectrometer

Analyte	m/z (Da)				
	Precursor	Transition 1	Transition 2	Transition 3	Transition 4
AcINH	180.0	121.0	138.1	79.0	52.1
desRIF	781.3	749.4	399.3	151.2	123.2
EMB	205.2	116.1	44.1	55.1	43.6
INH	138.1	79.1	52.1	93.0	51.6
PZA	124.0	81.1	54.1	79.0	52.0
RIF	823.3	151.0	123.2	163.1	106.9

Although the LC system was the same, peak shapes were altered on moving to the 3200 Q-trap. All analytes had split peaks and/or tailing. We speculated that this was due to hardware malfunction on the micro-LC system and decided to move the project to a normal-LC system. A Shimadzu Prominence LC system was available.

4.3. Shimadzu LC coupled to 3200 Q-trap

In contrast to micro-LC which utilizes flow rates less than 100 µl/min, normal-LC flow rates range from 100 µl/min to 10ml/min (84). An Agilent RP C18, 2.7µm particle size, 4.6mm internal diameter (ID) and 5cm long column was chosen. A flow rate of 400 µl/min and the gradient in Table 4.7 was chosen to start with. An initial chromatogram is presented in Figure 4.12.

Table 4.8: Initial gradient on the Shimadzu LC system

Time	% A	% B
0.00	80	20
0.10	80	20
0.75	20	80
3.25	20	80
3.75	80	20
5.00	80	20

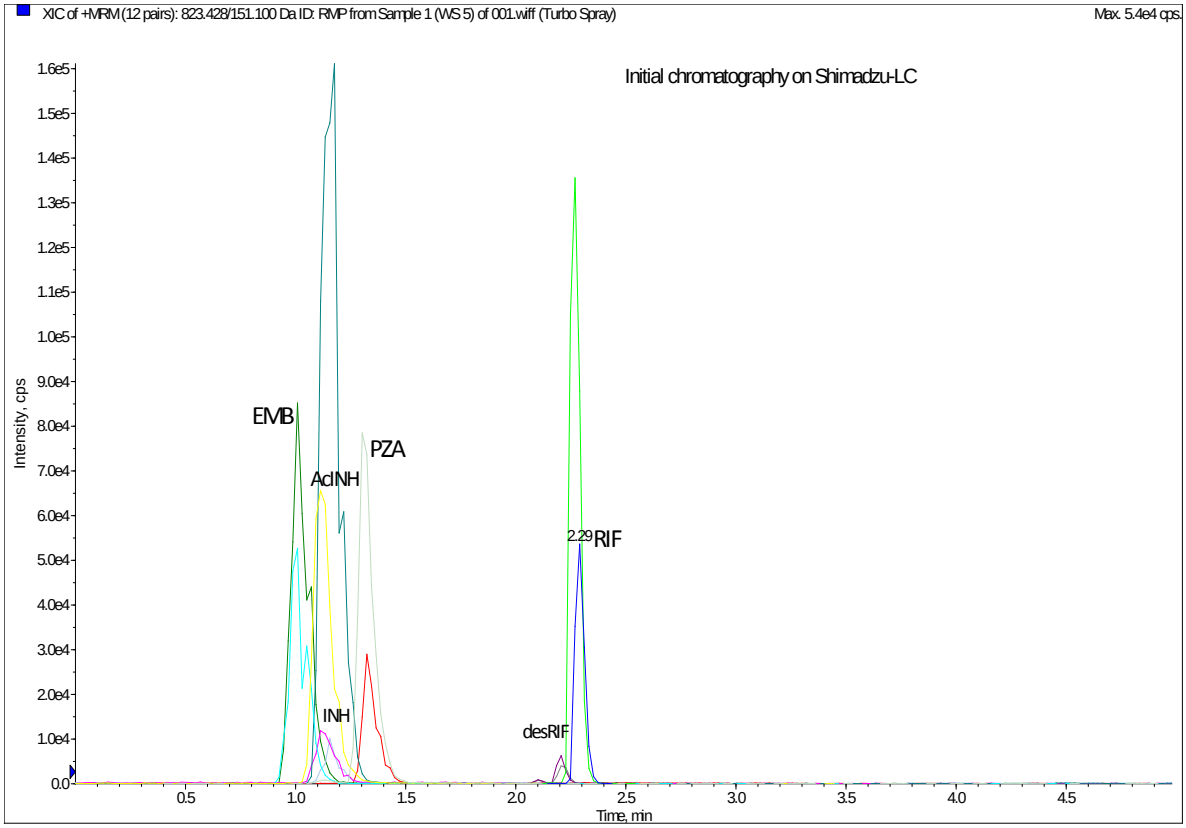

Figure 4.12: Initial chromatography on a Shimadzu LC system

Peak shapes were acceptable, but the polar analytes were not well separated, especially INH and its metabolite which were practically co-eluting. With MS as the detection method analytes do not have to separate completely as they are detected based on *m/z* ratio. However, cross talk may occur between analytes with fragment ions that have the same *m/z* value if the dwell time is short. Since separation was not critical at this stage, we decided to optimize source parameters, to ensure optimum sensitivity of all analytes. Figure 4.13 and Figure 4.14 show results from the optimization of temperature and IS voltage, respectively.

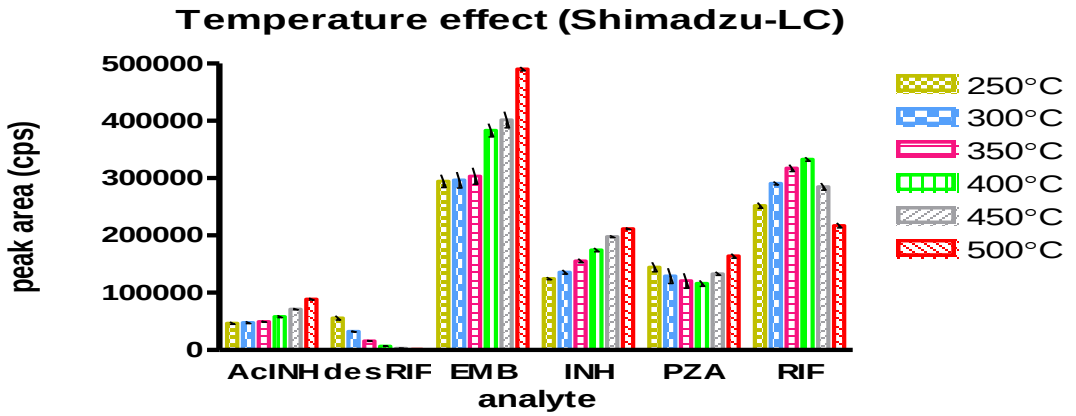

Figure 4.13: Effect of source temperature using normal-LC

INH, AcINH and EMB peak areas increased with increasing temperature while desRIF peak area decreased with increasing temperature. RIF had highest peaks at intermediate temperatures and PZA showed the opposite trend. Because desRIF peak areas were generally the lowest, and were the most sensitive to temperature changes, the optimum temperature for desRIF, 250^0C, was chosen to be the source temperature.

Results of IS voltage optimization are shown below. Although an IS voltage value of 3000 had the highest peak area for all analytes, the reproducibility was poor at this voltage and higher voltages were better for all analytes.

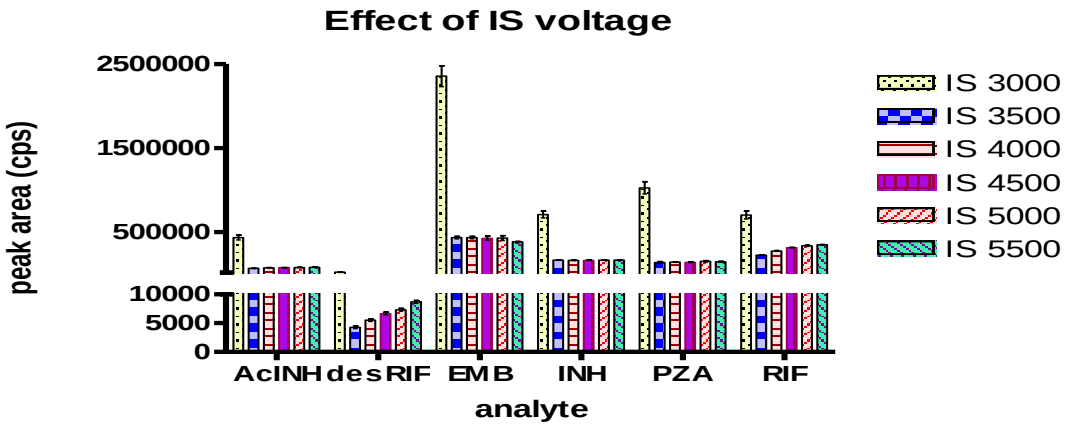

Figure 4.14: Effect of Ionspray voltage when using normal-LC

Sensitivity tests after optimization showed that sensitivity was poor for all analytes. The sensitivity and specificity experiments were done using plasma samples from six different sources. For sensitivity, plasma was spiked with working solution resulting in plasma with the lowest required concentration in the calibration range. For specificity, blank plasma samples were analysed. After sensitivity was found to be inadequate, precipitating solvent was optimized. Solvents tested were: acetonitrile, acetonitrile: dimethylsulphoxide (DMSO) 1:1 mixture and acetonitrile:methanol 1:1 mixture. Samples extracted using all solvents had low signal to noise ratios, especially for INH and AcINH. It was speculated that this was due to ion suppression by matrix components. The AcINH chromatogram in Figure 4.15 below suggests that this analyte was eluting concurrently with ion suppressing compounds and this was seen by the reduction in the noise intensity.

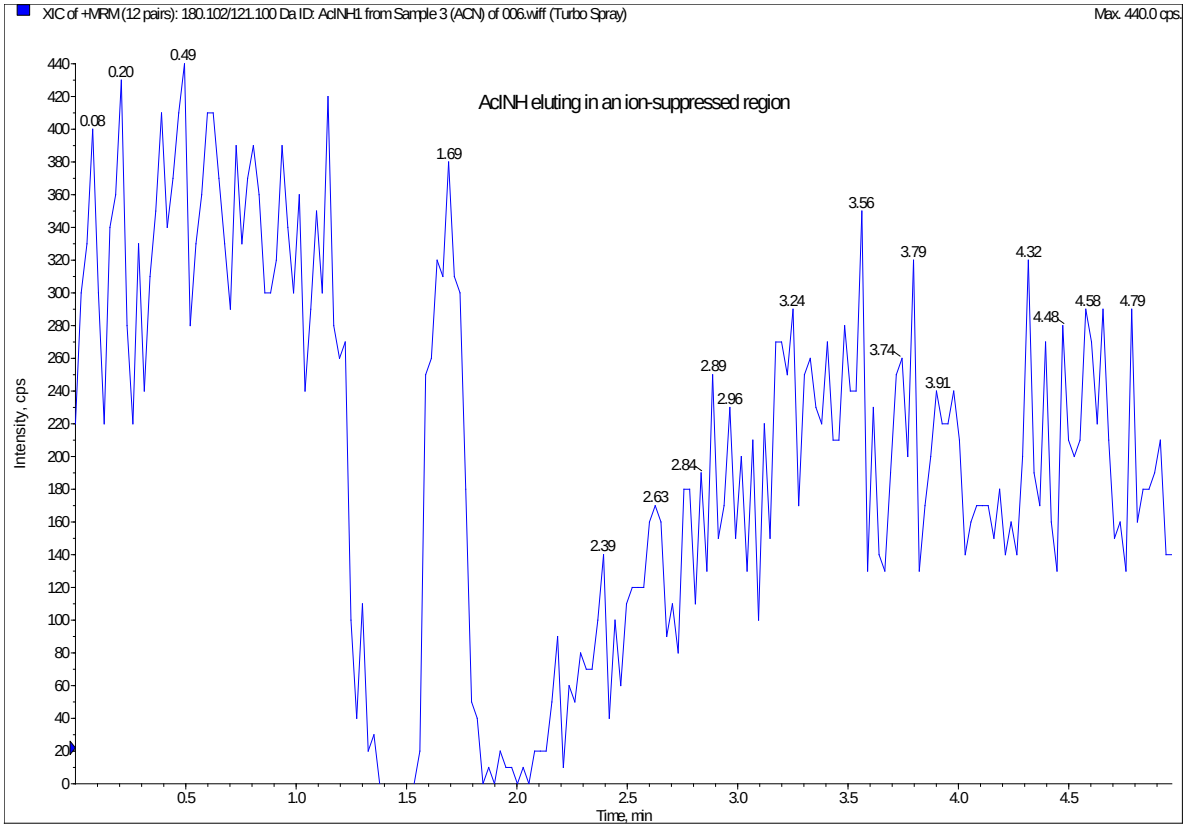

Figure 4.15: Chromatogram of AcINH eluting in an ion suppressed region

Proteins, phospholipids and other organic and inorganic ions cause ion suppression in LC-MS/MS analysis (85). Since the sample preparation method was protein precipitation, it was plausible to assume that the samples had very little proteins. Hence, phospholipids were suspected to be involved in causing matrix effects. Changing the extraction method can eliminate or reduce the effects of matrix components. A phospholipid removal step was incorporated using Phree phospholipid removal columns after protein precipitation. Signal to noise ratio was still low for INH, AcINH and desRIF. RIF had a high signal to noise ratio, but peaks were not as intense as those seen with protein precipitation only. The Phree phospholipid removal was possibly causing lower recovery of RIF. PZA and AcINH had higher signal intensity when Phree columns were used compared to when protein precipitation was used. INH did not show a significant difference. RIF and desRIF had higher

peak intensity with protein precipitation than when Phree was used. These analytes probably have some affinity for the Phree column hence the lower recovery. The use of Phree columns was discontinued because it did not result in the required sensitivity. Protein precipitation with Phree column extraction vs. protein precipitation data is presented in Figure 4.16.

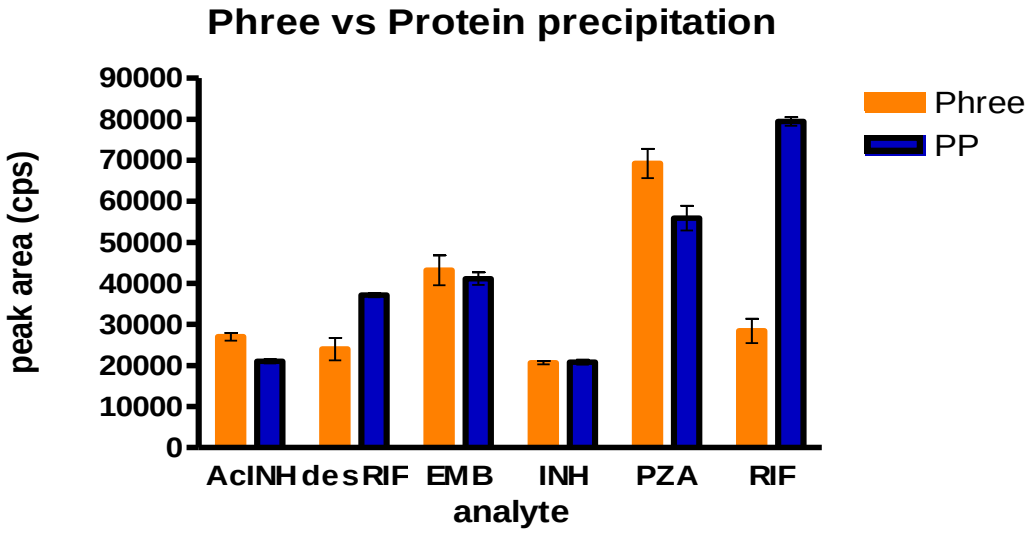

Figure 4.16: Protein precipitation versus protein precipitation with Phree column extraction

Source temperature and gas settings were altered with the hope of eliminating or reducing matrix effects. Modifying source parameters is usually the simplest strategy to mitigate matrix effects (74). However, this also failed to give satisfactory results. Chromatography was then modified by adjusting the gradient, resulting in better separation of the polar compounds, the most impressive of which was between INH and AcINH (presented in Figure 4.17). The run time also increased from 5 minutes to 6 minutes.

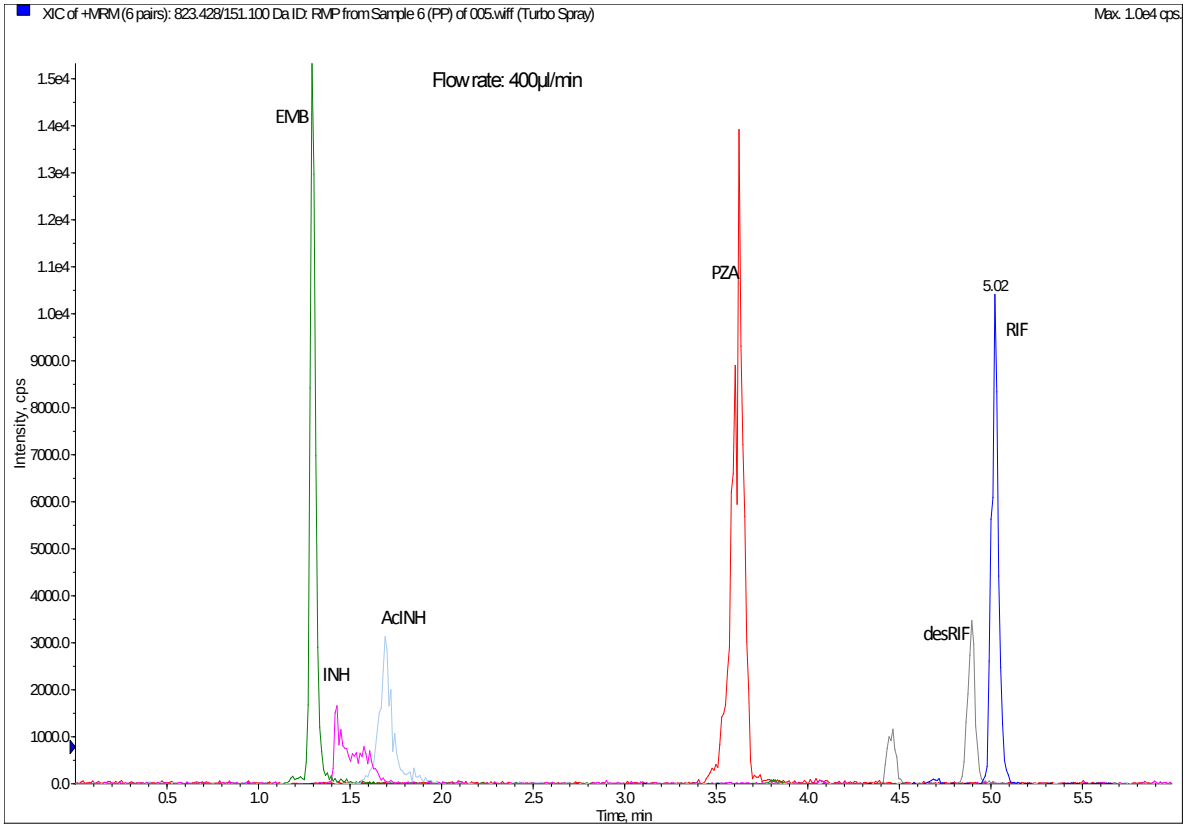

Figure 4.17: Chromatography after adjusting the gradient on the Shimadzu-LC

The flow program used was a multi-step gradient, at a constant flow rate of 400 µl/min, meant to increase separation between INH and AcINH without compromising peak intensities. Mobile phase A was an aqueous solution of 0.05% formic acid and mobile phase B was 0.05% formic acid in a 1:1 mixture of methanol and acetonitrile. The gradient profile is shown below in Table 4.9.

Table 4.9: Final gradient program on the Shimadzu-LC

Time (min)	% mobile phase A	% mobile phase B
0.0	95	5
1.4	95	5
1.8	45	55
3.5	10	90
3.7	10	90
2.8	30	70
4.5	95	5
6.0	95	5

Sensitivity for INH was still too low. Flow Injection Analysis (FIA) was performed to optimize source parameters, then a calibration curve check was done. While there was linearity for all analytes, variability was high, especially at lower concentrations. Injection of the same samples onto an AB Sciex API 4000, a more sensitive mass spectrometer, coupled to an Agilent 1200 series LC system showed lower variability, mostly within the required limits. It was thereafter decided that the project be moved to the more sensitive API 4000 mass spectrometer.

4.4. API 4000 – Agilent 1200

Infusion of 200 ng/ml solutions of all analytes and deuterated internal standards for each analyte was carried out to create an MS method. Methanol: water, 50:50

was used as the solvent. The infusion rate was 10 µl/min. The transitions monitored are shown in Table 4.10 and 4.11 for analytes and internal standards respectively.

Table 4.10: Product ions obtained after infusion on the API 4000 mass spectrometer

Analyte	Q1 m/z	product ion m/z
AcINH	180	121
desRIF	781	750
EMB	205	116
INH	138	79
PZA	124	81
RIF	824	792

Table 4.11: MRM transitions of internal standards

Internal standard	Q1 m/z	Product ion m/z
AcINH-d4	184	142
desRIF-d3	785	752
EMB-d4	209	120
INH-d4	142	83
PZA-15N,d3	128	84
RIF-d3	826	794

A simple gradient method was developed, with 0.05% formic acid in water solution as mobile phase A and 0.05% formic acid in a 1:1 mixture of methanol and acetonitrile as mobile phase B. An Agilent RP C18, 2.7µm particle size, 4.6mm internal diameter (ID) and 5cm long column was used. Reasonable separation was attained, although peak shapes were not impressive. The gradient is shown in Table 4.12.

Table 4.12: Initial gradient program on the Agilent 1200-LC system coupled to the API 4000 mass spectrometer

Step	Time(min)	Flow Rate(µl/min)	A (%)	B (%)
0	0	400	95	5
1	0.1	400	95	5
2	1	400	20	80
3	2	400	20	80
4	3	400	95	5
5	6	400	95	5

An initial chromatogram is presented in Figure 4.18.

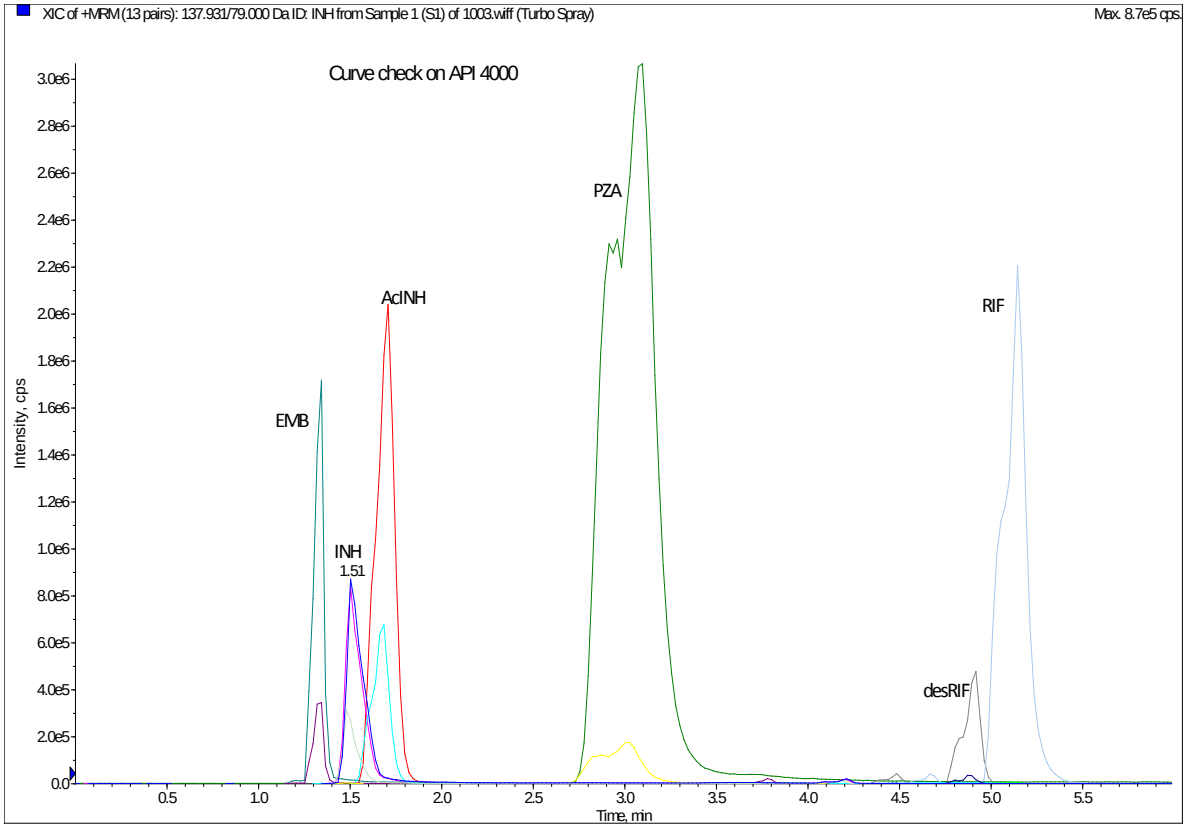

Figure 4.18: Initial chromatography on the Agilent 1200-LC system

We were not certain what was causing the peak "fronting" seen in Figure 4.22. This usually happens when the column is damaged or when the injection volume is too high causing a mass overload. Mass overload was unlikely considering the relatively large diameter of the column and the injection volume of 5 µl.

4.4.1. Extraction optimization

The protein precipitating solvent was optimized; peak intensities obtained using different precipitating solvents were compared. Mixtures of acetonitrile with DMSO and with methanol were compared. DMSO is a universal polar solvent, capable of dissolving polar and

non-polar compounds. The DMSO: acetonitrile mixture did not result in higher peak signal hence a simple 1:1 mixture of methanol and acetonitrile was chosen.

The supernatant obtained after protein precipitation, being highly organic, would result in poor peak shapes, as is usually the case when samples with a high organic content are injected into a highly aqueous environment. The supernatant, therefore had to be diluted by an aqueous component. Three aqueous phases; water, 0.05% formic acid solution and 0.05% acetic acid, were compared. INH, AcINH and PZA had the highest peak area when 0.05% acetic acid was used. EMB ionized best with 0.05% formic acid while desRIF preferred water. RMP had similar peak areas when water and when 0.05% formic acid was used, and 0.05% acetic acid gave lower intensities. Figure 4.19 shows the results for the six analytes.

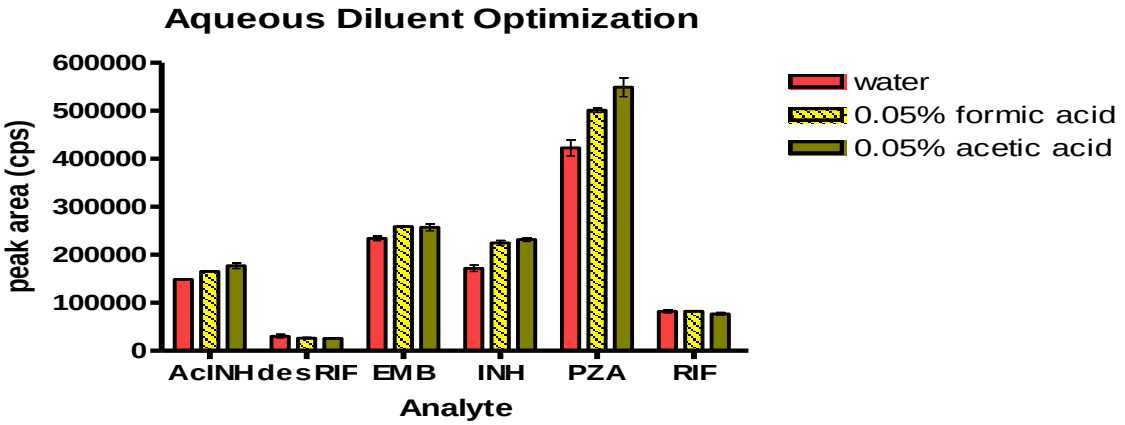

Figure 4.19: Aqueous diluent optimization

4.4.2. Effect of ascorbic acid

RIF, as well as desRIF, tended to show two peaks, as if two separate bands of the same compound would form in the chromatographic column. This phenomenon was prevented by adding ascorbic acid to the aqueous diluent. The first peak is possibly an oxidation product formed by the loss of two hydrogen atoms (86), which appears on the mass spectrum because the mass spectrometer may not be able to resolve the *m/z* values of the oxidized and non-oxidized species. However, this phenomenon is still poorly understood. The effect of ascorbic acid is demonstrated in the chromatograms in Figure 4.20 and 4.21.

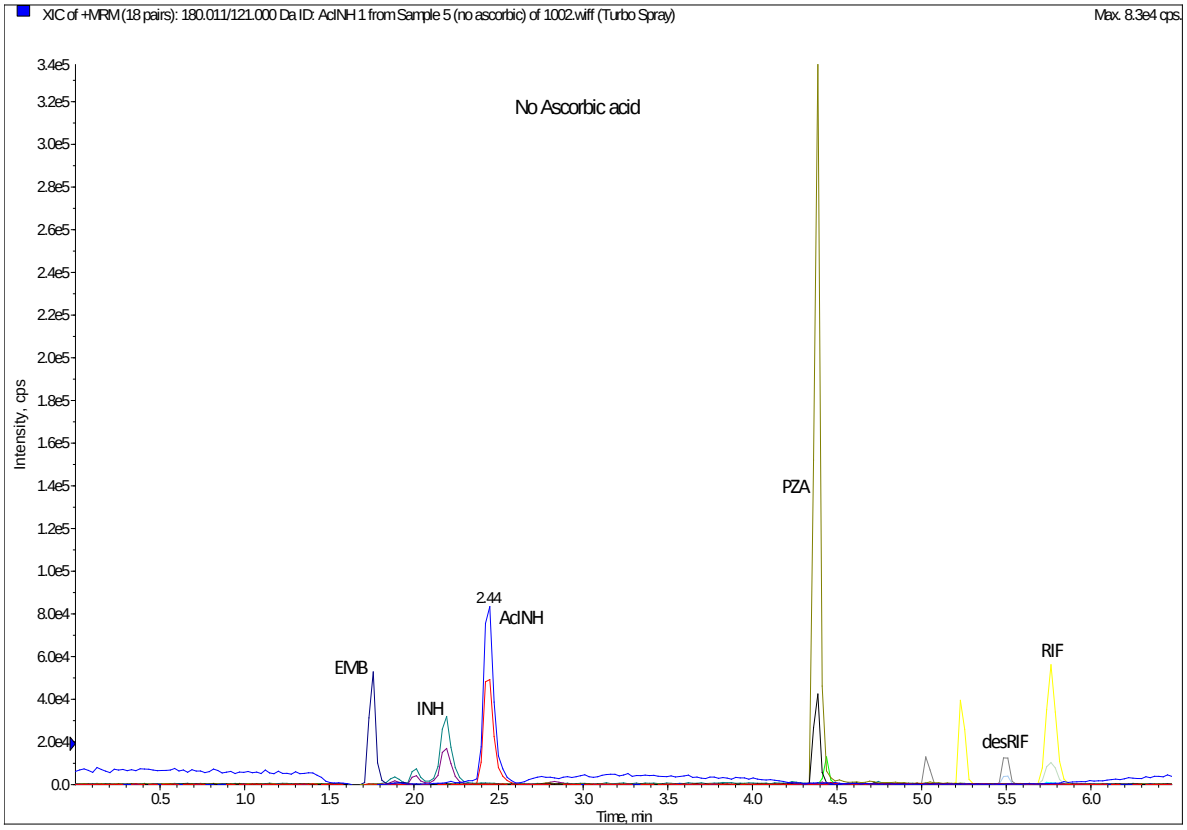

Figure 4.20: Chromatogram showing RIF (and desRIF) with two peaks in the absence of ascorbic acid

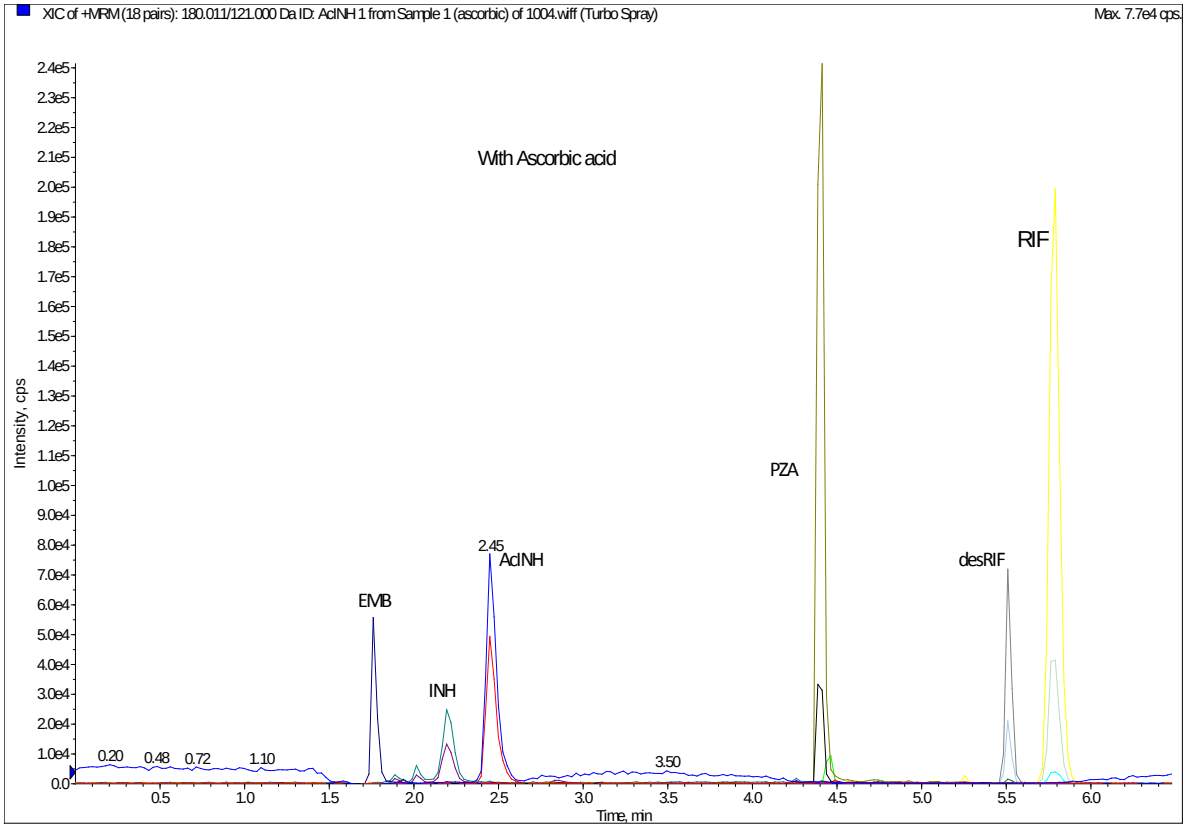

Figure 4.21: Chromatogram showing RIF (and desRIF) with a single peak in the presence of ascorbic acid

Ascorbic acid decreased peak areas of INH, AcINH, EMB and PZA and increased those of RIF and desRIF. The reproducibility was better for all analytes in the presence of ascorbic acid. Ultimately, addition of ascorbic acid was considered beneficial. The results are summarized in Table 4.13.

Table 4.13: Peak areas and coefficient of variability in the presence and in the absence of ascorbic acid

Analyte	No ascorbic acid (N=5)		with ascorbic acid (N=5)	
	Peak area mean (cps)	%CV	Peak area mean (cps)	%CV
AcINH	361200	2.8	319400	2.0
desRIF	40980	10.2	190800	3.7
EMB	166800	11.7	141400	4.9
INH	205800	5.2	149400	2.1
PZA	865200	5.7	730000	1.2
RIF	223000	5.7	885400	1.6

The low variability (when ascorbic acid is included) shown in Table 4.13 above indicated that the method was reproducible and thus ready for validation. Sensitivity and specificity were tested and were satisfactory. The chromatograms obtained at the lowest level of quantification and from blank plasma are shown in Figure 4.22 and Figure 4.23.

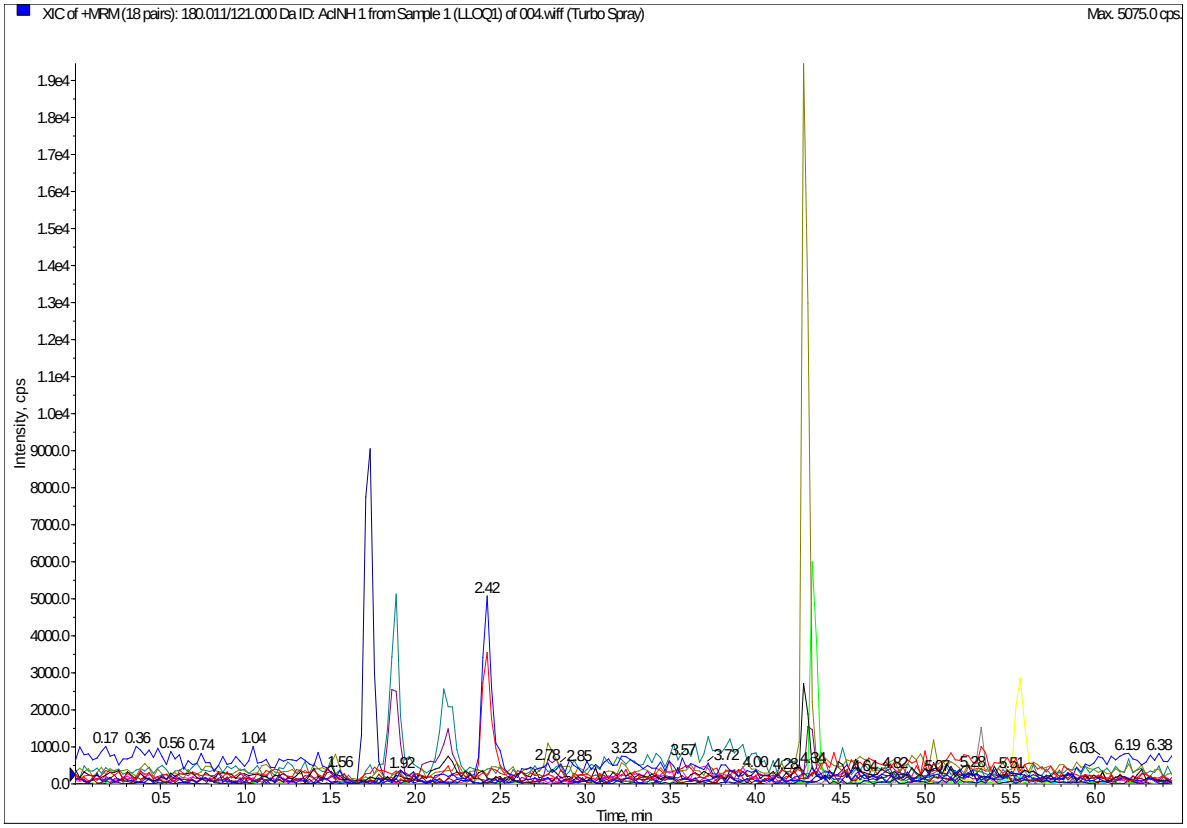

Figure 4.22: Chromatogram obtained at LLOQ

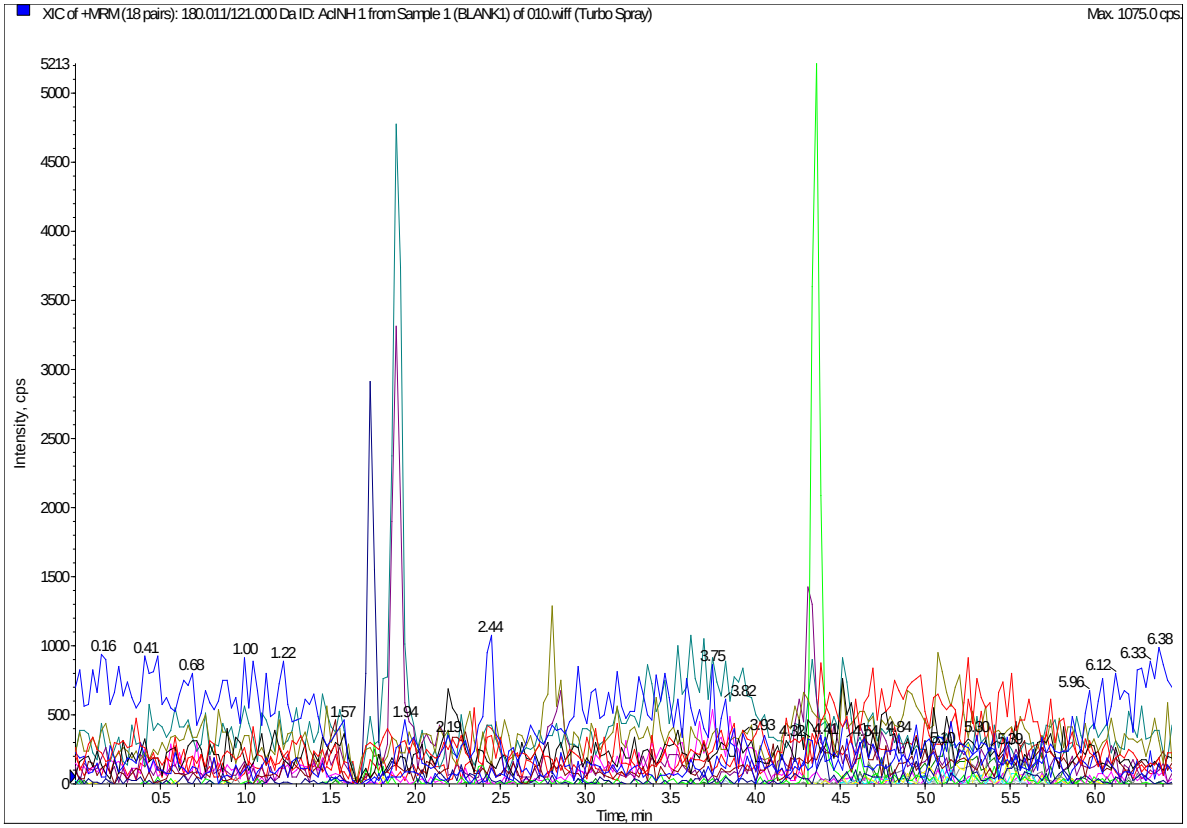

Figure 4.23: Chromatogram obtained when blank plasma was injected

The method was sensitive enough with signal to noise ratios above 5 for all analytes. The method was to be validated using protein precipitation as the sample preparation method. Two microliter injections would be made, and chromatographic separation achieved by gradient elution on an Agilent 1200 LC system and an AB Sciex API 4000 would be used for detection.

Chapter 5 : THE METHOD

This chapter gives details of the assay that is the final product of the method development process outlined in Chapter 4. This assay simultaneously quantified six compounds: AcINH, desRIF, EMB, INH, PZA and RIF, deuterated internal standards were used for each compound. The method used the LC-MS/MS technique and was validated on an AB-Sciex API 4000 triple quadrupole mass spectrometer, coupled to an Agilent 1200 liquid chromatography system. The calibration ranges and internal standards used for each analyte are presented in table 5.1.

Table 5.1: Analytes, calibration ranges and internal standards

Analyte	Calibration range (μg/ml)	LLOQ (μg/ml)	Internal standard
Rifampicin	0.120-30.0	0.120	Rifampicin-d3
25-des-rifampicin	0.040-10.0	0.040	25-des-rifampicin-d3
Isoniazid	0.100-25.0	0.100	Isoniazid-d4
N-acetyl-isoniazid	0.050-12.5	0.050	N-acetyl-isoniazid-d4
Pyrazinamide	0.320-80.0	0.320	Pyrazinamide-15N,d3
Ethambutol	0.020-5.00	0.020	Ethambutol-d4

5.1. Solution Preparation

5.1.1. Preparation of stock solutions

Stock solutions of analytes: RIF, desRIF, INH, AcINH, PZA and EMB, and internal standards: rifampicin-d3 (RIF-d3), 25-desacetyl-rifampicin-d3(desRIF-d3), isoniazid-d4(INH-d4), N-acetyl isoniazid-d4(AcINH-d4), pyrazinamide-15N,d3 (PZA-15N,d3) and ethambutol-d4 (EMB-d4) were prepared by weighing the analyte or internal standard into a container and dissolving this in the desired volume of solvent. The weighed mass of the analytes was adjusted where applicable (purity, salt, etc.). All stock solutions were kept at ~-80°C until required. Two stock solutions were prepared for each analyte by different analysts. These were analysed and compared to verify accuracy. These stock solutions were used to prepare working solutions or to spike blank biological matrix as required. Tables 5.2 to 5.13 show representative preparation of analyte and internal standard stock solutions

Table 5.2:Representative preparation of RIF stock solution

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted mass of analyte (mg)	SS1 Concentration (µg/ml)
Methanol	0.920	2.81	2.76	3000

* Reason for Adjustment (e.g. purity, salt, hydrate): Purity 98.2%

* Calculation:(2.81/100x98=2.760 mg)

Table 5.3: Representative preparation of desRIF stock solution

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 Concentration (µg/ml)
Methanol	0.430	1.32	1.29	3000

* Reason for Adjustment (e.g. purity, salt, hydrate): Purity 98%

* Calculation:(1.32/100x98=1.29mg)

Table 5.4: Representative preparation of RIF-d3 stock solution

Solvent used	Volume solvent (ml)	Weighed mass of ISTD (mg)	Adjusted mass of ISTD (mg)	ISS1 Concentration (µg/ml)
Methanol	0.500	0.500	0.500	1000

Table 5.5: Representative preparation of desRIF-d3 stock solution

Solvent used	Volume solvent (ml)	Weighed mass of ISTD (mg)	Adjusted mass of ISTD (mg)	ISS1 Concentration (µg/ml)
Methanol	1.00	1.00	1.00	1000

Table 5.6: Representative preparation of INH stock solution

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 Concentration (µg/ml)
Methanol	1.133	3.47	3.40	3000

* Reason for Adjustment (e.g. purity, salt, hydrate): Purity 98.0%

* Calculation: 3.47mg x 98.0/100 = 3.40 mg

Table 5.7: Representative preparation of AcINH stock solution

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 Concentration (µg/ml)
Methanol	1.167	3.57	3.50	3000

* Reason for Adjustment (e.g. purity, salt, hydrate): Purity 98.0 %

* Calculation: 3.57mg x 98.0/100 = 3.50 mg

Table 5.8: Representative preparation of INH-d4 stock solution

Solvent used	Volume solvent (ml)	Weighed mass of ISTD (mg)	Adjusted mass of ISTD (mg)	ISS1 Concentration (µg/ml)
Methanol	1.00	1.00	1.00	1000

Table 5.9: Representative preparation of AcINH-d4 stock solution

Solvent used	Volume solvent (ml)	Weighed mass of ISTD (mg)	Adjusted mass of ISTD (mg)	ISS1 Concentration (µg/ml)
Methanol	1.00	1.00	1.00	1000

Table 5.10: Representative preparation of PZA stock solution

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 Concentration (µg/ml)
Methanol	1.008	5.14	5.04	5000

* Reason for Adjustment (e.g. purity, salt, hydrate): Purity 98.0 %

* Calculation: 5.14 mg x 98.0/100 = 5.04 mg

Table 5.11: Representative preparation of PZA-^{15}N,d3

Solvent used	Volume solvent (ml)	Weighed mass of ISTD (mg)	Adjusted mass of ISTD (mg)	ISS1 Concentration (µg/ml)
Methanol	1.00	1.00	1.00	1000

Table 5.12: Representative preparation of EMB stock solution

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 Concentration (µg/ml)
Methanol	0.810	3.36	2.43	3000

* Reason for Adjustment (e.g. purity, salt, hydrate): Salt + Purity 98.0 %

* Calculation: 3.36 mg x 204.31/277.23 x 98.0/100 = 2.43 mg

Table 5.13: Representative preparation of EMB-d4 stock solution

Solvent used	Volume solvent (ml)	Weighed mass of ISTD (mg)	Adjusted mass of ISTD (mg)	ISS1 Concentration (µg/ml)
Methanol	1.00	1.00	1.00	1000

5.1.2. Preparation of Reference standard Working Solutions (WS)

The SS1 analyte stock solutions were used to prepare working solutions WS1 – WS8 in methanol containing concentrations of analytes as depicted in Table 5.15 (see Table 5.14 for spiking volumes used to prepare WS1 and WS11). These working solutions were used to spike individual calibration standards (Section 5.3). The SS2 analyte stock solutions were used to prepare working solutions WS11 – WS16 in methanol containing concentrations as depicted in Table 5.16. Working solutions WS11 - WS16 were used to prepare individually spiked quality control samples as well as a system suitability test sample (Section 5.4).

Table 5.14: Spiking volumes of stock solutions

Analyte	Concentration (µg/ml)	Solvent	Volume (µl) of SS1 spiked into WS1 and SS2 spiked into WS11
Acetyl Isoniazid	3000	Methanol	50.0
25-Desacetyl Rifampicin	3000	Methanol	40.0
Ethambutol	3000	Methanol	20.0
Isoniazid	3000	Methanol	100
Pyrazinamide	5000	Methanol	192
Rifampicin	3000	Methanol	120

Table 5.15: Preparation of working solutions used to prepare calibration standards

Working Solution (WS)	Blank solvent volume (µl)	Spiking solution	Spiking solution volume (µl)	Final WS volume (µl)	WS Concentration (µg/ml)					
					AcINH	Des-RIF	EMB	INH	PZA	RIF
WS1	78.0	SS1 x 6	50.0+40.0+ 20.0+100+ 192+120	600	250	200	100	500	1600	600
WS2	200	WS1	400	600	167	133	66.7	333	1067	400
WS3	200	WS2	200	400	83.3	66.7	33.3	167	533	200
WS4	300	WS3	200	500	33.3	26.7	13.3	66.7	213	80.0
WS5	300	WS4	200	500	13.3	10.7	5.33	26.7	85.3	32.0
WS6	300	WS5	200	520	5.33	4.27	2.13	10.7	34.1	12.8
WS7	300	WS6	180	480	2.00	1.60	0.80	4.00	12.8	4.80
WS8	300	WS7	300	600	1.00	0.800	0.400	2.00	6.40	2.40

Table 5.16: Preparation of working solutions used to prepare quality control samples

Working Solution (WS)	Blank solvent volume (µl)	Spiking solution	Spiking solution volume (µl)	Final WS volume (µl)	WS Concentration (µg/ml)					
					AcINH	Des-RIF	EMB	INH	PZA	RIF
WS11	228	SS x 6	50.0+40.0 + 20.0+100+ 192+120	750	200	160	80.0	400	1280	480
WS12	250	WS11	250	500	100	80.0	40.0	200	640	240
WS13	400	WS12	200	600	33.3	26.7	13.3	66.7	213	80.0
WS14	400	WS13	200	600	11.1	8.89	4.44	22.2	71.1	26.7
WS15	417	WS14	100	517	2.15	1.72	0.860	4.30	13.8	5.16
WS16	230	WS15	200	430	1.00	0.800	0.400	2.00	6.40	2.40

5.2. Preparation of calibration standards

Calibration standards were prepared by individually spiking blank plasma (anticoagulant K3EDTA) on crushed ice. A mixture of all analytes in working solutions (WS1 – WS8) was spiked into blank plasma to obtain the desired calibration standard concentrations (STD 1 - STD 8) as presented in Table 5.17. Multiple 120 µl aliquots of each calibration standard were stored in individual 1.5 ml polypropylene tubes and stored at approximately ~-80°C.

Table 5.17: Preparation of calibration standards

Working Solutions (WS)	Volume (µl) WS spiked into 1.90 ml plasma	Calibration Standard	Analyte Plasma Concentration (µg/ml)					
			A-INH	Des-RIF	EMB	INH	PZA	RIF
WS1	100	STD 1-ULOQ	12.5	10.0	5.00	25.0	80.0	30.0
WS2	100	STD 2	8.33	6.67	3.33	16.7	53.3	20.0
WS3	100	STD 3	4.17	3.33	1.67	8.33	26.7	10.0
WS4	100	STD 4	1.67	1.33	0.667	3.33	10.7	4.00
WS5	100	STD 5	0.667	0.533	0.267	1.33	4.27	1.60
WS6	100	STD 6	0.267	0.213	0.107	0.533	1.71	0.640
WS7	100	STD 7	0.100	0.0800	0.0400	0.200	0.640	0.240
WS8	100	STD 8 - LLOQ	0.0500	0.0400	0.0200	0.100	0.320	0.120

5.3. Preparation of quality controls

Quality control standards were prepared by individually spiking blank plasma (anticoagulant K3EDTA) on crushed ice. A mixture of all analytes in working solutions (WS11 – WS16) was spiked into blank plasma to obtain quality control standards at five concentration levels: QC Dil, QC High, QC Medium, QC Low and LLOQ as well as samples for system suitability checks (SYS 1) as depicted in Table 5.18.

Table 5.18: Preparation of quality control samples

Working Solutions (WS)	Volume (µl) WS spiked into 1.90 ml plasma	Quality Control	Analyte Plasma Concentration µg/ml					
			A-INH	Des-RIF	EMB	INH	PZA	RIF
WS11	100	QC H	10.0	8.00	4.00	20.0	64.0	24.0
WS12	100	QC M	5.00	4.00	2.00	10.0	32.0	12.0
WS13	100	SYS 1	1.67	1.33	0.667	3.33	10.7	4.00
WS14	100	SYS 2	0.556	0.444	0.222	1.11	3.56	1.33
WS15	100	QC L	0.107	0.0860	0.0430	0.215	0.688	0.258
WS16	100	LLOQ	0.0500	0.0400	0.0200	0.100	0.320	0.120
	Volume (µl) WS spiked into 0.90 ml plasma							
WS11	100	QC Dil	20.0	16.0	8.00	40.0	128	48.0

5.4. Verification of Calibration Standards and Quality Control Samples

The calibration standards and quality control samples were analysed in a batch prior to method validation to confirm their accuracy. Quality controls prepared independently by another analyst were used to verify the initial set of calibration standards. The independently prepared quality controls were within 85-115% of the target concentration, demonstrating no significant bias.

5.5. Preparation of Internal Standard Working Solution

The internal standard working solution (ISS2) was prepared by spiking appropriate volumes from each of the internal standard stock solutions (10.0 µl of ISS1-AcINH-d4, 10.0 µl of ISS1-desRIF-d3, 5.0 µl of ISS1-EMB-d4, 20.0 µl of ISS1-INH-d4, 40.0 µl of ISS1-PZA-15N,d3 and 25.0 µl of ISS1-RIF-d3) into 20.000 ml of acetonitrile: methanol mixture (1:1, v/v) to obtain the desired individual concentrations (refer to Table 5.19). . A volume of 200 µl of the internal standard working solution is added to each sample (excluding double blank samples), as described in section 5.7 of this chapter.

Table 5.19: Internal standard working solution

Working standard ID	Volume of ISS1 (µl)	Volume of ACN:MeOH (ml)	Concentration of internal standard in working solution (µg/ml)					
			A-INH	Des-RIF	EMB	PZA	INH	RIF
ISS2	10.0+10.0+ 5.0+20.0+40.0 +25.0	20.000	0.500	0.500	0.250	2.00	1.00	1.25

5.6. Buffers and other solutions

5.6.1. Mobile phase A: 0.05% Formic Acid in water:

1 litre water was added into a bottle, 0.5 ml formic acid was added, no pH adjustment was made, and the solution was stored at room temperature in normal light for a period of 2 weeks before being replaced. Helium was used to de-gas.

5.6.2. Mobile phase B: 0.05% Formic acid in acetonitrile: methanol (1:1, v/v).

500 ml acetonitrile was added to 500 ml methanol and 0.5 ml formic acid was added. This solution may be used within 1 week.

5.6.3. Autosampler needle wash: 50% methanol, (v/v).

500 ml methanol was added to 500ml water. This solution can be kept at room temperature for up to 2 weeks.

5.6.4. 0.05% formic acid in 25 µg/ml ascorbic acid solution.

1.25 mg ascorbic acid was dissolved in 50 ml water. 25 µl formic acid was added.

5.7. Extraction Procedure:

- An analytical batch was defined as follows: A single extraction procedure applied to a series of unknown samples, which must include a SYS sample (of sufficient volume to inject at least ten times), a minimum of eight calibration standards analysed in duplicate, three levels of quality controls analysed in six-fold, a blank and a double blank sample.
- Calibration standards were spread in duplicate throughout the run to capture instrument drift.
- Quality control samples were also spread in six-fold over the run to control the analysis appropriately.
- Double blanks and blanks were run after the highest calibration standard, consecutively to ascertain the level of carryover for the batch.

I. Plasma samples were thawed on crushed ice and vortexed briefly.50 µl plasma was aliquoted into 1.50 ml microcentrifuge tubes.

II. 200 µl of the internal standard working solution (acetonitrile: methanol (1:1, v/v) containing the internal standards, refer to Table 5.19 above) was added to the samples (not more than 4 to 8 samples at a time) and vortexed for 30 seconds. **NB: the double blank was extracted with an aliquot of internal standard-free acetonitrile: methanol (1:1, v/v).**

III. The samples were centrifuged at approximately 20 238 rcf for 5 minutes.

IV. 150 µl aliquots of an aqueous 0.05% formic acid in 25 µg/ml ascorbic acid solution (see section 5.7.4) were transferred into a 96-well plate.

V. 50 µl of the supernatant were added and mixed using the pipette

VI. 2 µl was injected onto the HPLC column.

VII. For dilutions: Only a 1:4 (sample: plasma, v/v) was validated. Dilutions were performed singly. The following procedure was used for performing dilution repeats:

VIII. 20 µl of sample (unknown or QC-dil) was pipetted into a labelled polypropylene tube.

IX. 80 µl of blank plasma was added to this sample and vortexed to mix.

X. 50 µl of this mix was pipetted into a clean labelled polypropylene microcentrifuge tube and extraction then proceeded as per normal.

5.7.1. Special Precautions: (e.g. waste diverting, column switching, washes, stability)

Samples were precipitated in groups of not more than 4 to 8 at a time. Some analytes (INH and AcINH) are temperature sensitive and all procedures were carried out on ice. On ice, ambient light stability has been shown for up to 4 hours for all analytes. The method was validated with a flow split at the MS source. A flow split is a tube which diverts some of the flow coming from the LC system and delivers it to waste to reduce the flow that enters the MS. Lower flow rates are associated with better sensitivity and less need to clean the MS.

5.7.2. Used Reagents, Chemicals, Consumables and Equipment

Reagents, chemicals, consumables and equipment required are listed in Tables 5.20 to 5.22.

Table 5.20: Reagents and chemicals

Reagent	Grade	Supplier
Formic acid	High purity	Merck
Acetonitrile	High purity	B&J Honeywell
Methanol	High purity	B&J Honeywell
Water	High purity	B&J Honeywell
Ascorbic acid	N/A	Sigma-Aldrich
Rifampicin reference material	N/A	USP
Rifampicin-d3 reference material	N/A	TRC
25-Desacetyl-Rifampicin reference material	N/A	TRC
25-Desacetyl-Rifampicin-d3 reference material	N/A	TRC
Isoniazid reference material	N/A	TRC
Isoniazid-d4 reference material	N/A	TRC
N-acetyl isoniazid reference material	N/A	TRC
N-acetyl isoniazid-d4 reference material	N/A	TRC
Pyrazinamide reference material	N/A	TRC
Pyrazinamide-15N,d3 reference material	N/A	TRC
Ethambutol reference material	N/A	TRC
Ethambutol-d4 reference material	N/A	TRC

Table 5.21: Necessary consumables

Description	**Supplier**
96 well plates	Agilent
Sealing mats	Agilent
Microcentrifuge tubes	Lasec SA
Analytical Column: Agilent C18, 2.7µm, 50 x 4.6 mm	Agilent
Pipette tips (white)	LASEC
Pipette tips (yellow)	LASEC
Pipette tips (blue)	LASEC

Table 5.22: Equipment required

Name	Model	Manufacturer
Vortex	Vortex Genie 2	Scientific Industries
Centrifuge	5424	Eppendorf
Pipette: 2-20 µl	Various	Various
Pipette: 20 – 200 µl	Various	Various
Pipette: 200-1000 µl	Various	Various
Sartorius CPA2P Micro balance	CPA2P	Carl Zeiss (Pty Ltd)
Sonicator	703	Labotec (Pty Ltd)

5.8. Instrument and chromatographic conditions

The instrument used, and chromatographic conditions are presented in Table 5.23

Table 5.23: Instrument and chromatographic conditions

Instrument used	API4000
Project	2017-12 TB Drugs
Acquisition method	Multiplex_first-lineTBdrugs.dam
Analytical Column	Agilent, 2.7µ, C18, 50 x 4.6 mm
Column Temperature	~30°C
Mobile Phase and Flow Gradient	Mobile phase A: 0.05% formic acid in water Mobile phase B: 0.05% formic acid in 1:1 Acetonitrile:Methanol mixture (v/v) Time (minutes) %Mobile phase A %Mobile phase B Flow rate (µl/min) 0.00 100 0.00 300 0.10 100 0.00 300 1.00 20.0 80.0 300 2.50 20.0 80.0 300 2.55 20.0 80.0 400 3.50 100 0.00 400 6.00 100 0.00 400 6.10 100 0.00 300
Pump Type	Agilent 1200 Quaternary Pump
Autosampler Type	Agilent 1200
Sample arrangement	96-well plate
Injection Volume	2 µl
Autosampler Temperature	~8°C

5.8.1. System Suitability Requirements

The system suitability test was done before each validation batch, a SYS sample was injected at least 10 times and the acceptance criteria was that the peak area ratio of the last 6 injected samples must be below 5%.

5.9. Detection details

5.9.1. Detection settings

The mass spectrometer used for detection and mode of detection are presented in Table 5.24.

Table 5.24:Detection settings

Mass Spectrometer Identity	API4000
APCI/ESI	ESI

5.9.2. Electrospray ionization settings

The settings in the electrospray ion source are presented in Table 5.25.

Table 5.25: Ion source parameter settings

Nebuliser gas (Gas 1) (arbitrary unit)	40
Turbo gas (Gas 2) (arbitrary unit)	60
CUR (curtain gas) (arbitrary unit)	40
CAD (collision gas) (arbitrary unit)	10
TEM (Source Temperature) (°C)	300
IS (Ion Spray Voltage) (V)	5000

5.9.3. MS/MS settings

The MS/MS settings used for each analyte are presented in Table 5.26.

Table 5.26: MS/MS settings

	RIF	Des-RIF	RIF-d3	Des-RIF-d3	INH	INH-d4	AcINH	AcINH-d4	PZA	PZA-^{15}N,d3	EMB	EMB-d4
Protonated molecular ion mass (m/z) $[M+H]^+$	823.4	781.5	826.4	784.5	137.9	142.0	180.0	184.0	124.0	127.9	205.1	209.1
Product ion mass (m/z) Quantifier	791.6	749.5	794.4	752.4	79.1	83.1	121.0	142.1	81.0	84.0	116.1	120.1
Product ion mass (m/z) Qualifier	151.1	399.3			93.1		138.0		79.0		81.0	
Dwell time (ms)	80	80	80	80	80	80	80	80	80	80	80	80
Declustering potential (V)	91	66	86	71	51	51	61	61	51	51	51	46
Entrance potential (V)	10	10	10	10	10	10	10	10	10	10	10	10
Collision energy (eV)	25	19	27	19	39	41	31	23	25	25	21	23
Collision cell exit potential (V)	24	22	22	20	6	6	10	12	6	6	10	10

5.9.4. Scan description

The scan type, polarity and pause time are presented in Table 5.27.

Table 5.27: Scan description

Scan Type	MRM
Polarity	Positive
Pause Time (ms)	5

5.9.5. Spectra

Analyte: RIF

The following is a mass spectrum of rifampicin after collision induced dissociation in the fragmentation cell, showing the RIF precursor ion at m/z 823 as well as the product ions.

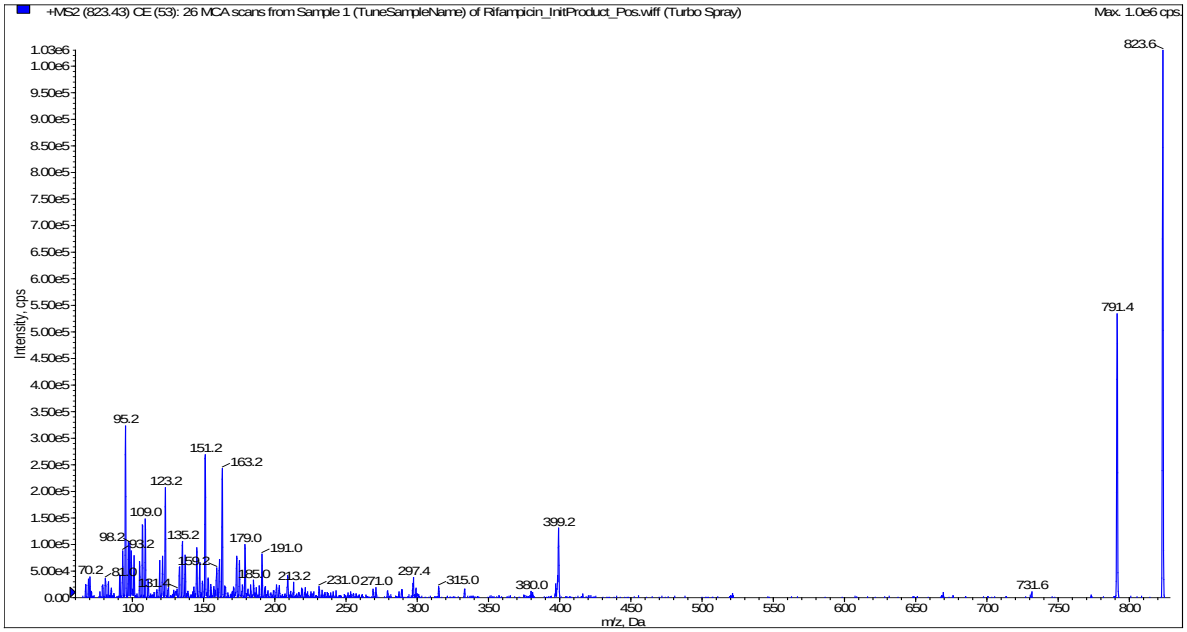

Figure 5.1: RIF mass spectrum

Internal standard: RIF-d3

The following is a mass spectrum of RIF-d3 after collision induced dissociation in the fragmentation cell, showing the RIF-d3 precursor ion at m/z 826 as well as the product ions.

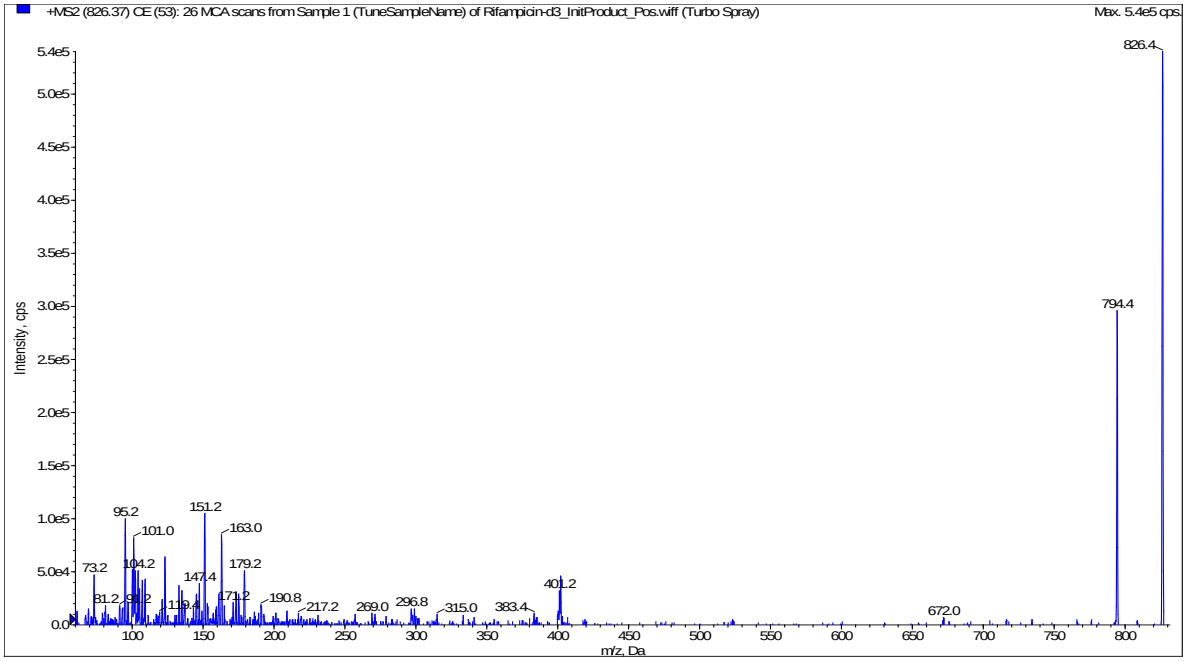

Figure 5.2: RIF-d3 mass spectrum

Analyte: DesRIF

The following is a mass spectrum of desRIF after collision induced dissociation in the fragmentation cell, showing the desRIF precursor ion at m/z 781 as well as the product ions.

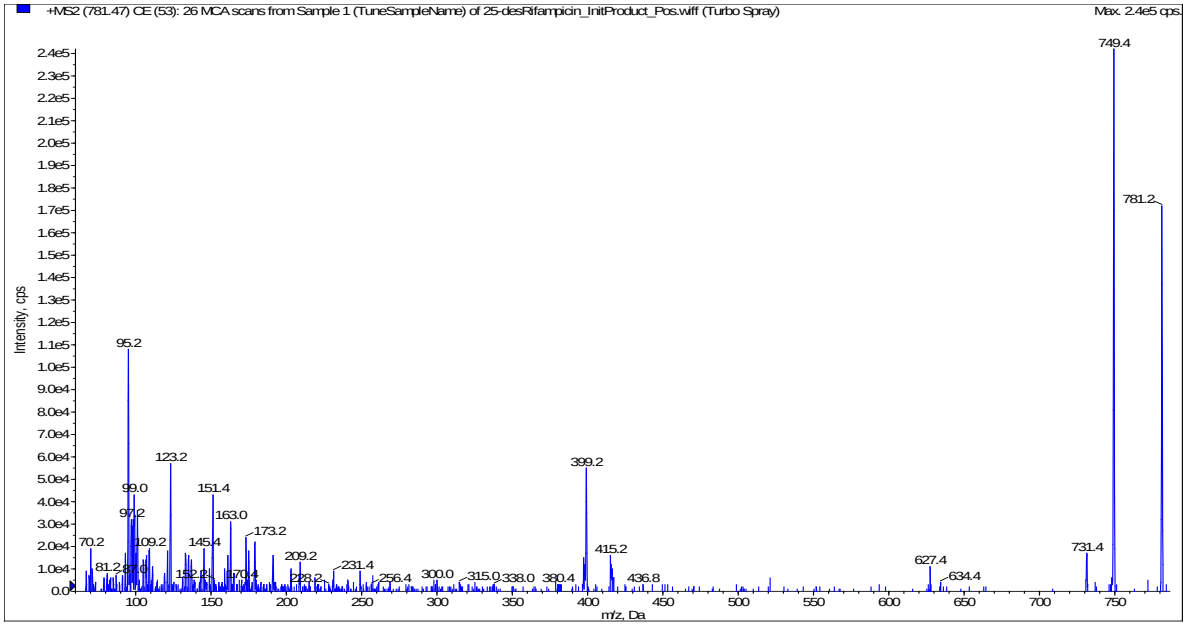

Figure 5.3: DesRIF mass spectrum

Internal standard: DesRIF-d3

The following is a mass spectrum of desRIF-d3 after collision induced dissociation in the fragmentation cell, showing the desRIF-d3 precursor ion at m/z 784 as well as the product ions.

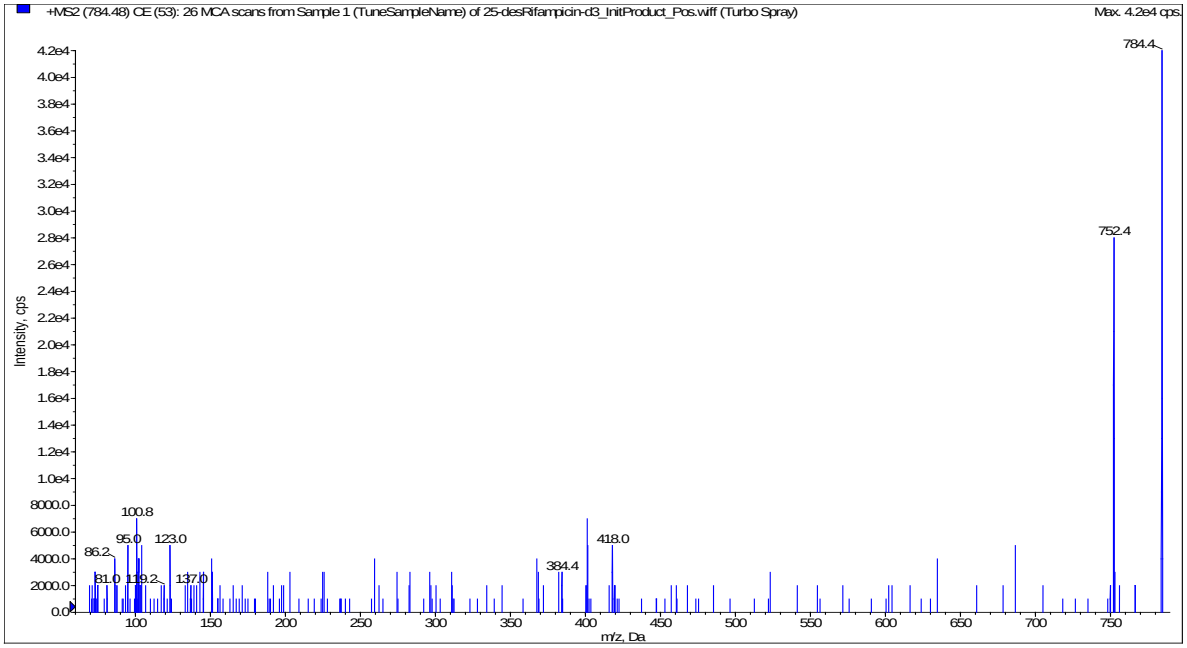

Figure 5.4: DesRIF-d3 mass spectrum

Analyte: INH

The following is a mass spectrum of INH after collision induced dissociation in the fragmentation cell, showing the INH precursor ion at m/z 138 as well as the product ions.

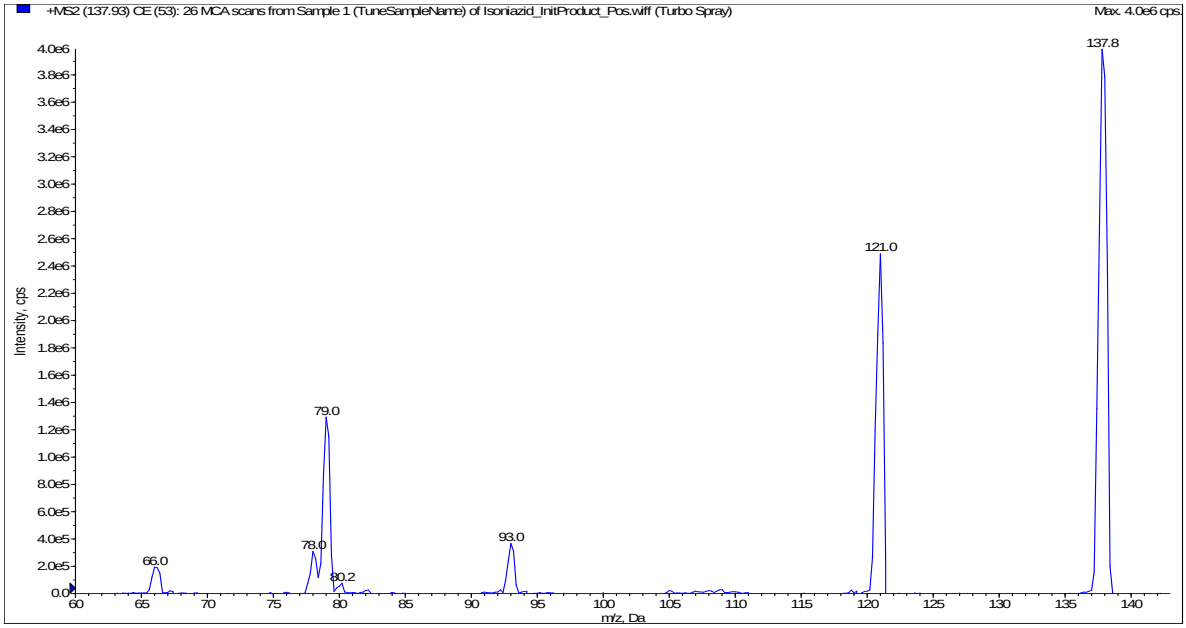

Figure 5.5: INH mass spectrum

Internal standard: INH-d4

The following is a mass spectrum of INH-d4 after collision induced dissociation in the fragmentation cell, showing the INH-d4 precursor ion at m/z 142 as well as the product ions.

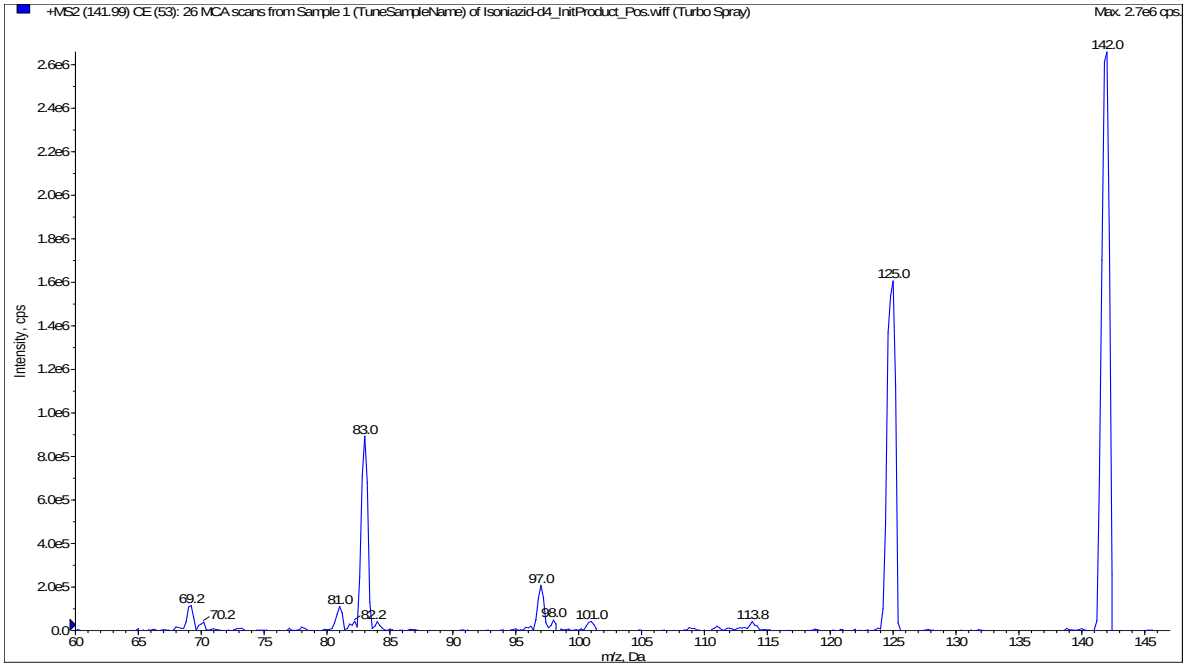

Figure 5.6: INH-d4 mass spectrum

Analyte: AcINH

The following is a mass spectrum of AcINHsoniazid after collision induced dissociation in the fragmentation cell, showing the AcINH precursor ion at m/z 180 as well as the product ions.

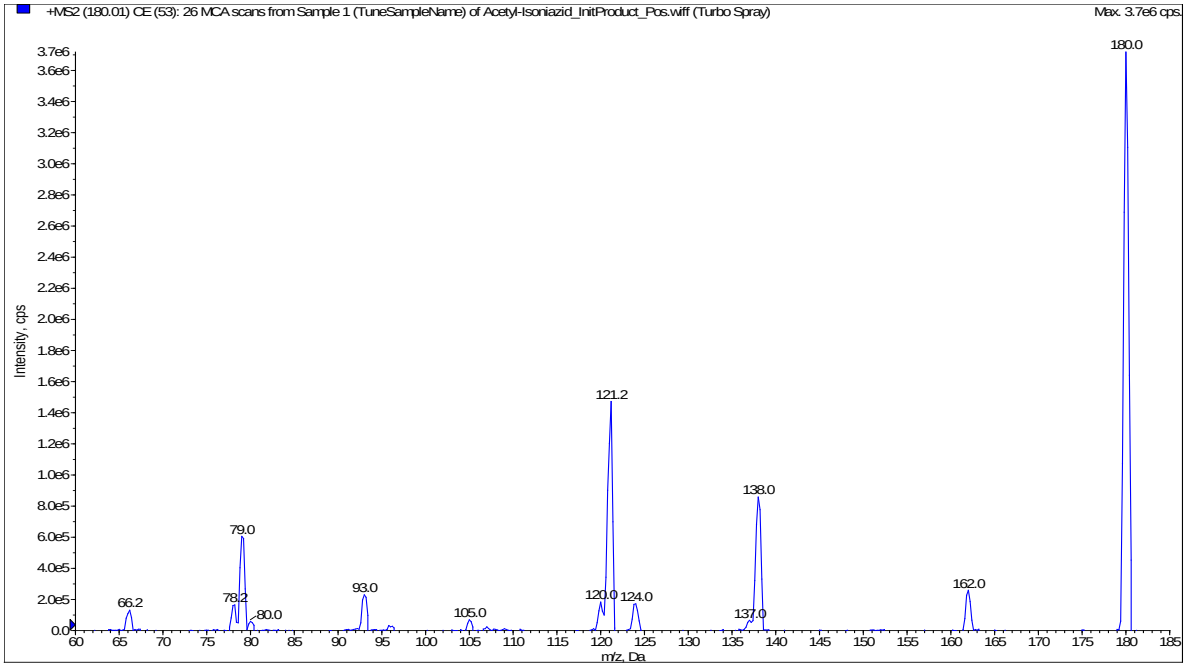

Figure 5.7: AcINH mass spectrum

Internal standard: AcINH-d4

The following is a mass spectrum of AcINH-d4 after collision induced dissociation in the fragmentation cell, showing the AcINH-d4 precursor ion at m/z 184 as well as the product ions.

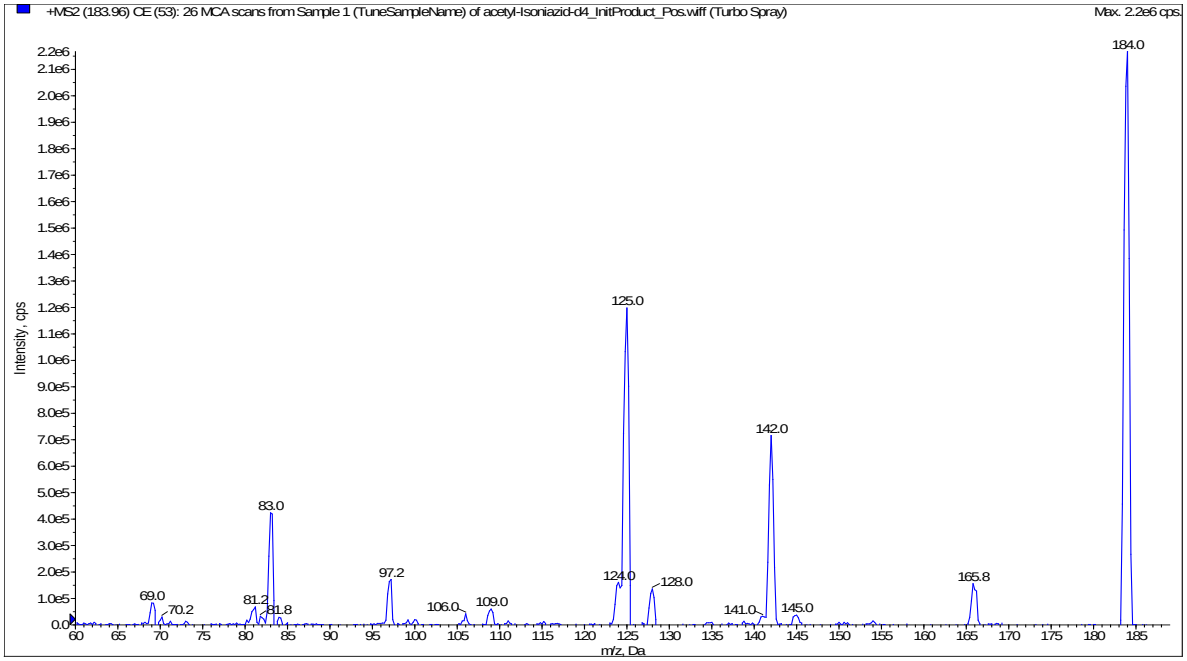

Figure 5.8: AcINH-d4 mass spectrum

Analyte: PZA

The following is a mass spectrum of the analyte PZA after collision induced dissociation in the fragmentation cell, showing the PZA precursor ion at m/z 124 as well as the product ions.

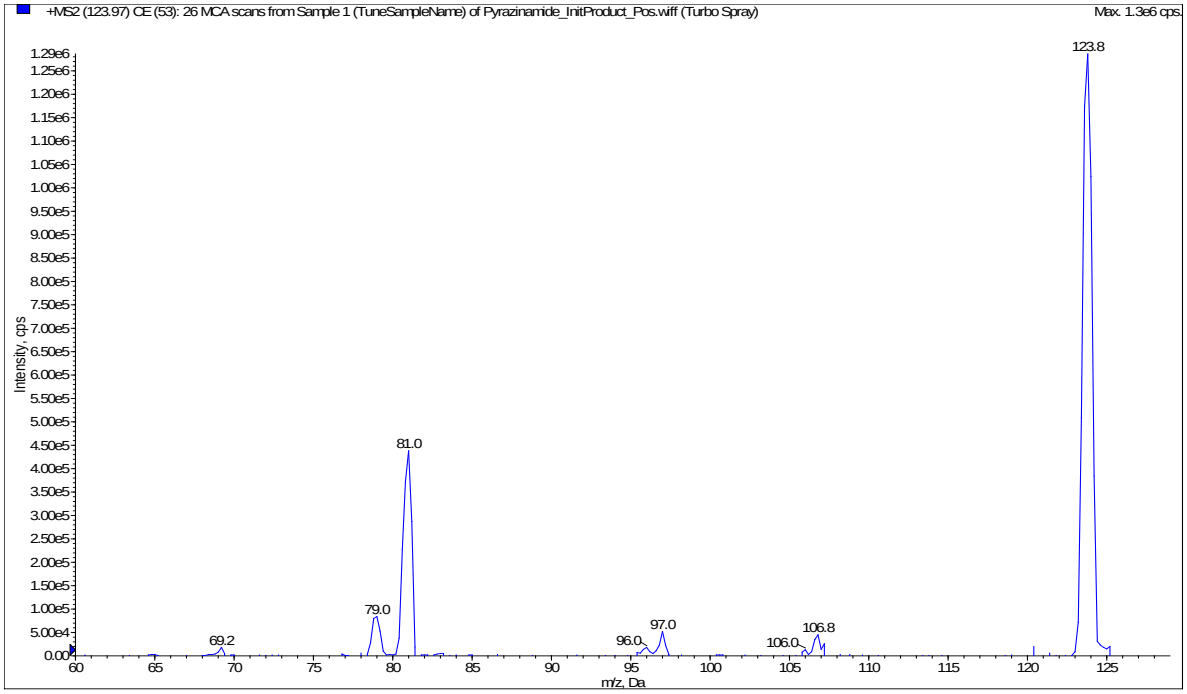

Figure 5.9: PZA mass spectrum

Internal standard: PZA-^{15}N,d3

The following is a mass spectrum of the internal standard PZA-^{15}N,d3 after collision induced dissociation in the fragmentation cell, showing the PZA-^{15}N,d3 precursor ion at m/z 128 as well as the product ions.

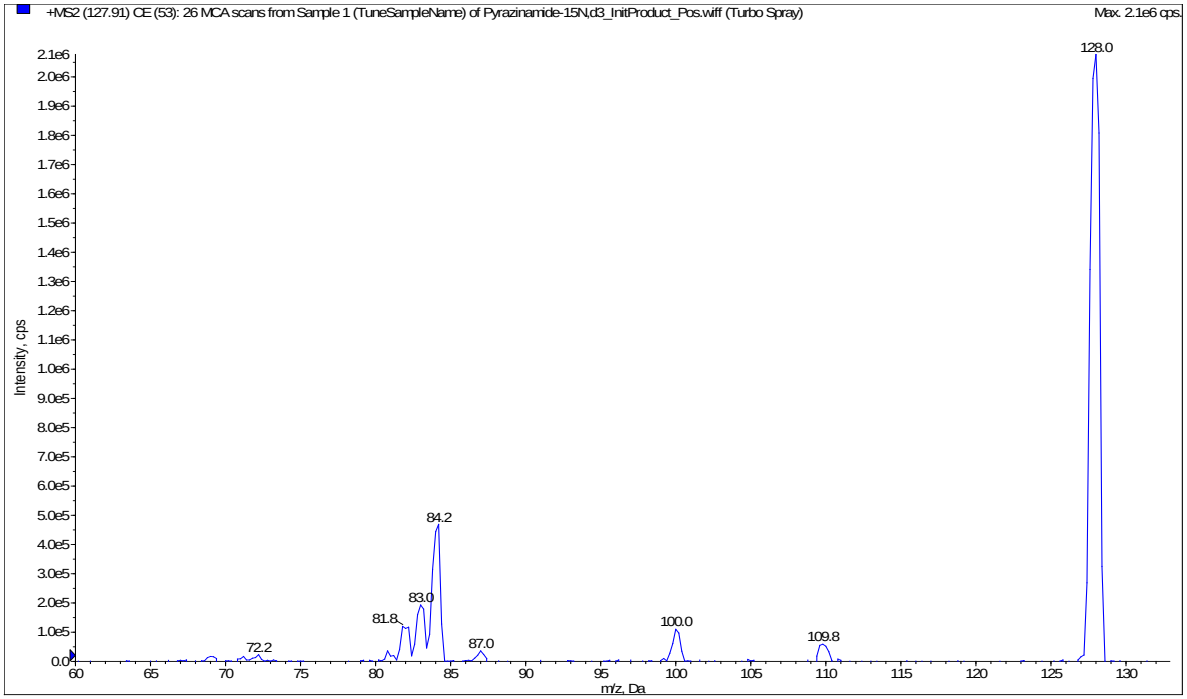

Figure 5.10: PZA-^{15}N,d3 mass spectrum

Analyte: EMB

The following is a mass spectrum of the analyte EMB after collision induced dissociation in the fragmentation cell, showing the EMB precursor ion at m/z 205 as well as the product ions.

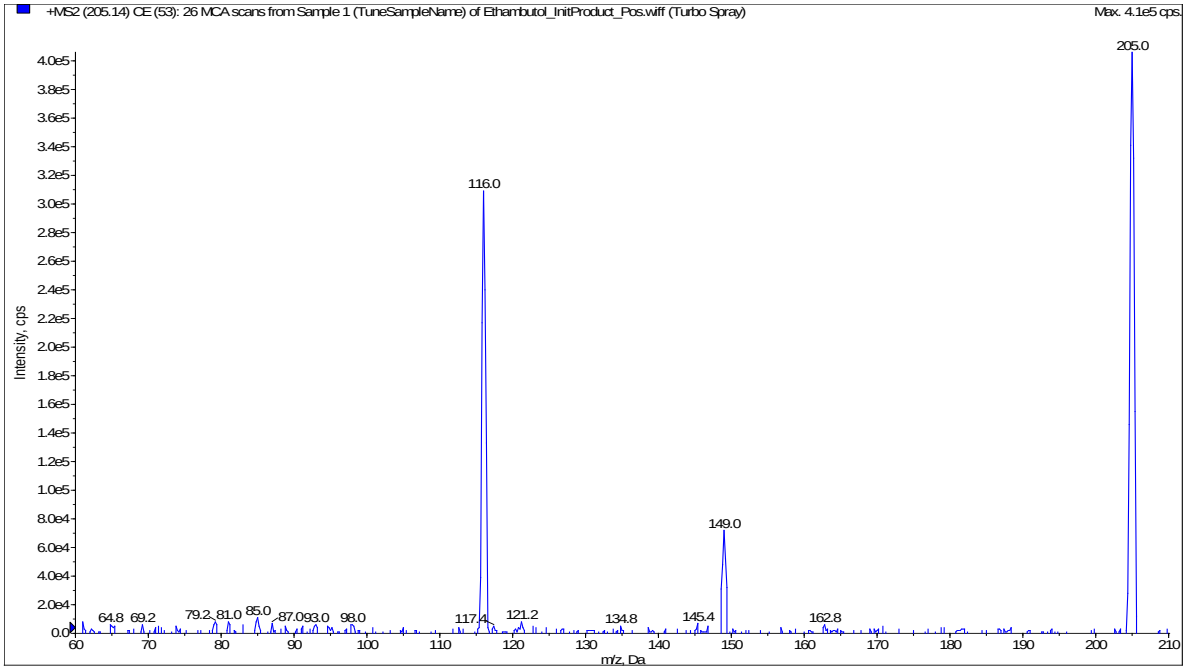

Figure 5.11: EMB mass spectrum

Internal standard: EMB-d4

The following is a mass spectrum of the internal standard EMB-d4 after collision induced dissociation in the fragmentation cell, showing the EMB-d4 precursor ion at m/z 209.3 as well as the product ions.

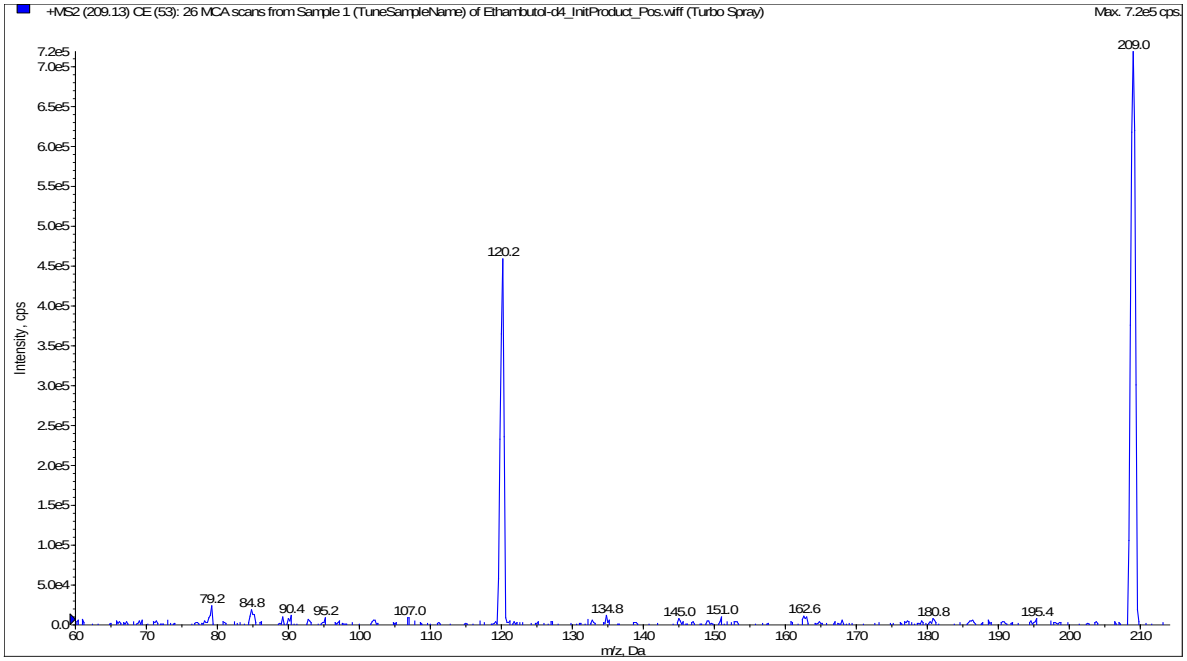

Figure 5.12: EMB-d4 mass spectrum

5.10. Recording and integration

5.10.1. Quantitation parameters

Quantitation parameters are presented in Table 5.28.

Table 5.28: Quantitation parameters

	Analyte: RIF	**ISTD: RIF-d3**	**Analyte: 25-Des-RIF**	**ISTD: 25-Des-RIF-d3**	**Analyte: INH**	**ISTD: INH-d4**	**Analyte: AcINH**	**ISTD: AcINH-d4**	**Analyte: PZA**	**ISTD: PZA-15N,d3**	**Analyte: EMB**	**ISTD: EMB-d4**
Software	Analyst 1.6.2											
Algorithm	Analyst Classic											
Calibration Fit Type	Quadratic		Quadratic		Quadratic		Quadratic		Quadratic		Quadratic	
Parameter	Area ratio		Area ratio		Area ratio		Area ratio		Area ratio		Area ratio	
Curve Weighting	1/x		1/x		1/x		1/x		$1/x^2$		1/x	
Bunching factor	1	1	1	1	1	1	1	1	1	1	1	1
Number of smoothes	5	5	5	5	5	5	5	5	5	5	5	5

5.10.2. Precursor and product ions monitored

All precursor and product ions monitored (analytes and internal standards) are presented in Table 5.29.

Table 5.29: Precursor and product ions monitored

	Precursor Ion Mass (amu)	Product Ion Mass (amu)
Analyte 1: RIF	823.4	791.6
ISTD 1: RIF-d3	826.3	794.4
Analyte 2: DesRIF	781.5	749.5
ISTD 2: DesRIF-d3	784.5	752.4
Analyte 3: INH	137.9	79.1
ISTD 3: INH-d4	142.0	83.1
Analyte 4: AcINH	180.0	121.0
ISTD 4: AcINH-d4	184.0	142.1
Analyte 5: PZA	124.0	81.0
ISTD 5: PZA-15N,d3	127.9	84.0
Analyte 6: EMB	205.1	116.1
ISTD 6: EMB-d4	209.1	120.1

5.10.3. Retention times

Approximate retention times for all analytes and internal standards are presented in Table 5.30.

Table 5.30: Retention times

Analyte:	Time (min)
RIF	~ 5.7
DesRIF	~ 5.4
RIF-d3	~5.7
DesRIF-d3	~ 5.4
INH	~2.2
AcINH	~2.4
INH-d4	~2.2
AcINH-d4	~2.4
PZA	~4.3
PZA-15N,d3	~4.3
EMB	~1.7
EMB-d4	~1.7

5.11. Acceptance criteria

5.11.1. Calibration standards

75% of the standards used must fall within 15% of the nominal concentration (i.e. 85-115%), except for the LLOQ, which should be within 20% (i.e. 80-120%) of the nominal value. The %CV for duplicate observations should be less than 15% for all points, except for the LLOQ which may display a 20% CV. Duplicate points (and not averages) are used to construct the

calibration curve. Failed points must be excluded from the calibration curve regression determination. Should both values fail to meet the acceptance criteria, then this point should not be used to construct the calibration curve and must be excluded from the experiment. This may necessitate a reanalysis of the unknown samples contained in the failed run. Goodness of fit criteria should be monitored but are not the ideal way of assessing the validity of a calibration curve.

5.11.2. Quality control

A minimum of four quality control levels (2 x high (~80% of the ULOQ), 2 x medium, 2 x low (~3 x LLOQ) and 2 x LLOQ) are run with every analytical batch. Two thirds of the quality controls analysed must meet the acceptance criteria of 85-115% accuracy and less than 15% CV. The allowable failures may not be of the same concentration, i.e. 50% of controls run at every level must pass. Failure to meet these acceptance criteria will necessitate a reanalysis of the unknown samples. Additional quality controls may be run to ensure against a failed batch owing to failed ULOQ or LLOQ standards.

Chapter 6 : VALIDATION

Validation is the process of proving that an analytical method is accurate and precise, thus ensuring the integrity of results produced. Essential parameters to ensure the acceptability of the performance of a bioanalytical method are accuracy, precision, selectivity, sensitivity, reproducibility and stability (87). Linearity of calibration curves, matrix effects and recovery are also frequently assessed. This chapter outlines the validation experiments that were conducted, meeting the requirements of the American Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (88, 89).

6.1. Accuracy and precision and reproducibility

Accuracy is expressed as a percentage of the observed concentration over the nominal concentration (% Accuracy). It must be within 15% over the entire calibration curve and can be within 20% at the LLOQ. Precision is expressed as the coefficient of variation (% CV) and must also be within 15% over the entire calibration range and within 20% at the LLOQ. Three validation batches were run on separate days to demonstrate both within-run and inter-occasion accuracy and precision. Each batch had eight calibration standards, STD1 to STD8, with concentrations covering the calibration range (Chapter 5, Table 5.17) and quality control standards at four concentration levels: LLOQ, QCL, QCM and QCH (Chapter 5, Table 5.18), to ensure the accuracy of the calibration standards and the precision of the method. A quality control sample above the limit of quantification (QC Dil) was diluted with plasma four times its volume and analysed to prove that samples with concentrations above the limits of quantification can be diluted and accurately assayed. The calibration standards and quality

controls were previously prepared as outlined in Chapter 5 and stored at -80^{0}C. Calibration standards were analysed in duplicate and quality control standards were analysed in six replicates in each validation batch.

AcINH

The overall accuracy and precision of calibration standards and QCs for AcINH are summarised in Tables 6.1 and 6.2.

Table 6.1: Overall Summary of Calibration Standard Accuracy and Precision: Validation 1-3: AcINH

	Sample ID	**STD 8 - LLOQ**	**STD 7**	**STD 6**	**STD 5**	**STD 4**	**STD 3**	**STD 2**	**STD 1**
	Nominal Conc.	**0.05 µg/ml**	**0.1 µg/ml**	**0.27 µg/ml**	**0.67 µg/ml**	**1.67 µg/ml**	**4.17 µg/ml**	**8.33 µg/ml**	**12.5 µg/ml**
Validation 1	**Data Point 1**	0.0566	0.100	*	0.624	1.54	4.32	8.65	12.0
	Data Point 2	0.0550	0.100	0.242	0.631	1.71	4.20	8.20	12.9
Validation 2	**Data Point 1**	0.0514	0.0976	0.258	0.665	1.68	4.26	8.06	12.2
	Data Point 2	0.0529	0.105	0.253	0.654	1.68	4.23	8.37	12.9
Validation 3	**Data Point 1**	0.0564	0.111	0.260	0.672	1.63	4.26	8.13	12.4
	Data Point 2	0.0410	0.102	0.257	0.689	1.65	4.15	8.38	12.7
	N	6 of 6	6 of 6	5 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
	Mean	0.0522	0.103	0.254	0.656	1.65	4.24	8.30	12.5
	Standard Dev.	0.00586	0.00491	0.00727	0.0248	0.0593	0.0567	0.216	0.377
	%CV	**11.2**	**4.8**	**2.9**	**3.8**	**3.6**	**1.3**	**2.6**	**3.0**
	Accuracy	**104.4**	**102.6**	**94.1**	**97.9**	**98.7**	**101.6**	**99.6**	**100.1**

*Failed to meet acceptance criteria

Table 6.2: Overall Quality Control Accuracy and Precision Estimation: AcINH

	Sample ID	**LLOQ**	**QCL**	**QCM**	**QCH**	**QC DIL**
	Nominal Conc.	**0.0500**	**0.107**	**5.00**	**10.0**	**20.0**
		(µg/ml)	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**
	Replicates	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**
Validation 1	**Data Point #1**	0.0490	0.0898	4.38	10.2	18.5
	Data Point #2	0.0472	0.0871	4.38	9.70	18.2
	Data Point #3	0.0486	0.102	4.73	10.1	18.7
	Data Point #4	0.0570	0.104	4.55	10.4	18.0
	Data Point #5	0.0470	0.0952	4.63	10.1	18.9
	Data Point #6	0.0455	0.0994	4.78	10.2	17.8
Validation 2	**Data Point #1**	0.0407	0.100	5.48	10.6	
	Data Point #2	0.0491	0.0973	5.44	10.6	
	Data Point #3	0.0531	0.0917	5.17	10.8	
	Data Point #4	0.0493	0.0936	5.27	10.8	
	Data Point #5	0.0484	0.0946	5.32	11.2	
	Data Point #6	0.0434	0.0875	5.20	10.7	
Validation 3	**Data Point #1**	0.0505	0.0990	5.15	10.8	
	Data Point #2	0.0551	0.0993	5.27	10.4	
	Data Point #3	0.0532	0.0962	5.16	11.0	
	Data Point #4	0.0535	0.107	5.11	10.4	
	Data Point #5	0.0456	0.105	5.26	10.5	
	Data Point #6	0.0487	0.106	5.37	10.8	
	N	18 of 18	18 of 18	18 of 18	18 of 18	6 of 6
	Mean	0.0492	0.0974	5.04	10.5	18.4
	Standard Dev.	0.00412	0.00603	0.361	0.372	0.424
	%CV	**8.4**	**6.2**	**7.2**	**3.5**	**2.3**
	Accuracy	**98.3**	**91.1**	**100.7**	**105.1**	**91.8**

desRIF

The overall accuracy and precision of calibration standards and QCs for desRIF are summarised in Tables 6.3 and 6.4.

Table 6.3: Overall Summary of Calibration Standard Accuracy and Precision: Validation 1-3: desRIF

	Sample ID	**STD 8 - LLOQ**	**STD 7**	**STD 6**	**STD 5**	**STD 4**	**STD 3**	**STD 2**	**STD 1**
	Nominal Conc.	**0.0400**	**0.0800**	**0.213**	**0.533**	**1.33**	**3.33**	**6.67**	**10.0**
		µg/ml	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**
Validation 1	**Data Point #1**	0.0416	0.0800	0.192	0.568	1.30	3.19	7.27	9.00
	Data Point #2	0.0480	0.0700	0.187	0.523	1.51	3.23	6.30	11.3
Validation 2	**Data Point #1**	0.0402	0.0875	0.199	0.514	1.36	3.29	6.59	10.8
	Data Point #2	0.0384	0.0860	0.193	0.546	1.35	3.35	6.72	9.40
Validation 3	**Data Point #1**	0.0426	*	0.214	0.504	1.30	3.03	6.94	11.1
	Data Point #2	0.0384	0.0780	0.217	0.528	1.43	3.57	6.44	9.10
	N	6 of 6	5 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
	Mean	0.0415	0.0810	0.200	0.531	1.37	3.28	6.71	10.1
	Standard Dev.	0.0384	0.0702	0.187	0.504	1.30	3.03	6.3	8.96
	%CV	**8.6**	**8.6**	**6.2**	**4.3**	**6**	**5.4**	**5.3**	**10.6**
	Accuracy	**103.8**	**100.6**	**94.1**	**99.6**	**103.3**	**98.4**	**100.6**	**101.2**

*Failed to meet acceptance criteria

Table 6.4: Overall Quality Control Accuracy and Precision Estimation: desRIF

	Sample ID	**LLOQ**	**QCL**	**QCM**	**QCH**	**QC DIL**
	Nominal Conc.	**0.0400**	**0.0860**	**4.00**	**8.00**	**16.0**
		(µg/ml)	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**
	Replicates	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**
Validation 1	**Data Point #1**	0.0372	0.0791	3.48	8.93	15.0
	Data Point #2	0.0377	0.0848	3.53	8.40	14.5
	Data Point #3	0.0387	0.0848	4.14	7.63	15.6
	Data Point #4	0.0450	0.0990	4.24	8.84	14.3
	Data Point #5	0.0377	0.0789	3.67	8.13	16.9
	Data Point #6	0.0410	0.0972	4.02	8.50	15.2
Validation 2	**Data Point #1**	0.0320	0.0882	4.11	7.67	
	Data Point #2	0.0359	0.0872	4.01	7.30	
	Data Point #3	0.0441	0.0791	3.74	8.63	
	Data Point #4	0.0323	0.0706	4.05	11.4*	
	Data Point #5	0.0383	0.0776	4.13	8.46	
	Data Point #6	0.0301	0.0775	3.99	8.89	
Validation 3	**Data Point #1**	0.0510	0.0821	3.60	7.74	
	Data Point #2	0.0412	0.0869	3.66	6.95	
	Data Point #3	0.0564	0.0914	3.78	8.27	
	Data Point #4	0.0511	0.0764	3.61	8.96	
	Data Point #5	0.0384	0.0903	3.84	8.20	
	Data Point #6	0.0348	0.0683	3.38	8.38	
	N	18 of 18	18 of 18	18 of 18	18 of 18	6 of 6
	Mean	0.0402	0.0833	3.83	8.40	15.2
	Standard Dev.	0.00706	0.00829	0.261	0.947	0.934
	%CV	**17.6**	**9.9**	**6.8**	**11.3**	**6.1**
	Accuracy	**100.4**	**96.9**	**95.8**	**105.1**	**95.3**

EMB

The overall accuracy and precision of calibration standards and QCs for EMB are summarised in Tables 6.5 and 6.6

Table 6.5: Overall Summary of Calibration Standard Accuracy and Precision: Validation 1-3: EMB

	Sample ID	**STD 8 – LLOQ**	**STD 7**	**STD 6**	**STD 5**	**STD 4**	**STD 3**	**STD 2**	**STD 1**
	Nominal Conc.	**0.0200**	**0.0400**	**0.107**	**0.267**	**0.667**	**1.67**	**3.33**	**5.00**
		µg/ml	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**
Validation 1	**Data Point #1**	0.0232	0.0380	*	0.273	0.603	1.59	3.36	4.80
	Data Point #2	0.0184	*	0.100	0.297	0.651	1.71	3.51	5.09
Validation 2	**Data Point #1**	0.0223	0.0387	0.0993	0.246	0.675	1.70	3.18	4.91
	Data Point #2	0.0213	0.0436	0.100	0.251	0.655	1.71	3.48	5.07
Validation 3	**Data Point #1**	0.0213	0.0431	0.0980	0.253	0.630	1.60	3.33	4.82
	Data Point #2	0.0202	0.0380	0.0991	0.302	0.666	1.81	3.28	5.20
	N	6 of 6	5 of 6	5 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
	Mean	0.0211	0.0403	0.0994	0.271	0.647	1.69	3.36	4.98
	Standard Dev.	0.00166	0.00283	0.001	0.0246	0.0264	0.0809	0.124	0.162
	%CV	**7.8**	**7.0**	**1.0**	**9.1**	**4.1**	**4.8**	**3.7**	**3.3**
	Accuracy	**105.7**	**100.7**	**92.9**	**101.3**	**97.0**	**100.9**	**100.8**	**99.6**

*Failed to meet acceptance criteria

Table 6.6: Overall Quality Control Accuracy and Precision Estimation: EMB

	Sample ID	**LLOQ**	**QCL**	**QCM**	**QCH**	**QC DIL**
	Nominal Conc.	**0.0200**	**0.0430**	**2.00**	**4.00**	**8.00**
		(µg/ml)	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**
	Replicates	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**
Validation 1	**Data Point #1**	0.0180	0.0384	1.71	3.98	8.10
	Data Point #2	0.0184	0.0411	1.70	3.98	7.17
	Data Point #3	0.0201	0.0461	1.88	3.85	7.90
	Data Point #4	0.0178	0.0474	2.05	4.51	7.83
	Data Point #5	0.0210	0.0425	2.00	4.10	7.98
	Data Point #6	0.0197	0.0387	1.87	4.08	7.52
Validation 2	**Data Point #1**	0.0196	0.0370	2.16	4.04	
	Data Point #2	0.0209	0.0397	2.16	4.19	
	Data Point #3	0.0203	0.0366	2.06	4.49	
	Data Point #4	0.0202	0.0412	2.07	4.14	
	Data Point #5	0.0219	0.0429	2.08	4.30	
	Data Point #6	0.0201	0.0384	1.96	4.28	
Validation 3	**Data Point #1**	0.0196	0.0357	1.86	4.41	
	Data Point #2	0.0198	0.0365	2.01	4.36	
	Data Point #3	0.0177	0.0383	2.05	4.27	
	Data Point #4	0.0180	0.0395	2.07	4.07	
	Data Point #5	0.0198	0.0420	2.23	4.33	
	Data Point #6	0.0176	0.0406	2.11	4.03	
	N	18 of 18	18 of 18	18 of 18	18 of 18	6 of 6
	Mean	0.0195	0.0401	2.00	4.19	7.75
	Standard Dev.	0.00127	0.00321	0.148	0.188	0.344
	%CV	**6.5**	**8.0**	**7.4**	**4.5**	**4.4**
	Accuracy	**97.4**	**93.3**	**100.1**	**104.7**	**96.9**

INH

The overall accuracy and precision of calibration standards and QCs for INH are summarised in Tables 6.7 and 6.8.

Table 6.7: Overall Summary of Calibration Standard Accuracy and Precision: Validation 1-3: INH

	Sample ID	**STD 8 - LLOQ**	**STD 7**	**STD 6**	**STD 5**	**STD 4**	**STD 3**	**STD 2**	**STD 1**
	Nominal Conc.	**0.100**	**0.200**	**0.533**	**1.33**	**3.33**	**8.33**	**16.7**	**25**
		µg/ml	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**
Validation 1	**Data Point #1**	0.106	0.191	*	1.42	3.04	8.37	17.2	24.8
	Data Point #2	*	*	0.503	1.38	3.39	8.26	16.6	25.0
Validation 2	**Data Point #1**	0.106	0.191	0.396	1.42	3.04	8.37	17.2	24.8
	Data Point #2	0.130	0.248	0.503	1.38	3.39	8.26	16.6	25.0
Validation 3	**Data Point #1**	0.119	*	0.489	1.27	3.11	8.25	16.7	24.5
	Data Point #2	0.109	0.192	0.491	1.32	3.37	8.45	17.5	25.0
	N	5 of 6	4 of 6	5 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
	Mean	0.110	0.199	0.495	1.32	3.25	8.39	16.9	24.9
	Standard Dev.	0.00523	0.00979	0.00782	0.0681	0.16	0.163	0.409	0.2
	%CV	**4.7**	**4.9**	**1.6**	**5.2**	**4.9**	**1.9**	**2.4**	**0.8**
	Accuracy	**110.2**	**99.3**	**92.8**	**99.1**	**97.5**	**100.7**	**101.2**	**99.4**

*Failed to meet acceptance criteria

Table 6.8: Overall Quality Control Accuracy and Precision Estimation: INH

	Sample ID	**LLOQ**	**QCL**	**QCM**	**QCH**	**QC DIL**
	Nominal Conc.	**0.100**	**0.215**	**10.0**	**20.0**	**40.0**
		(µg/ml)	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**
	Replicates	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**
Validation 1	**Data Point #1**	0.0815	0.163	8.26	20.3	39.4
	Data Point #2	0.0826	0.176	8.55	19.6	38.8
	Data Point #3	0.108	0.188	9.85	20.4	38.2
	Data Point #4	0.0986	0.185	9.74	20.1	37.5
	Data Point #5	0.104	0.209	9.66	19.9	37.4
	Data Point #6	0.113	0.205	9.32	20.0	39.2
Validation 2	**Data Point #1**	0.0694	0.181	10.2	21.5	
	Data Point #2	0.107	0.208	10.5	20.8	
	Data Point #3	0.113	0.195	10.3	20.4	
	Data Point #4	0.105	0.202	10.2	22.0	
	Data Point #5	0.0872	0.229	10.8	21.4	
	Data Point #6	0.110	0.189	10.4	21.0	
Validation 3	**Data Point #1**	0.115	0.200	10.3	21.3	
	Data Point #2	0.110	0.243	10.3	20.9	
	Data Point #3	0.0900	0.178	10.8	21.3	
	Data Point #4	0.109	0.183	10.3	21.0	
	Data Point #5	0.106	0.243	10.9	21.3	
	Data Point #6	0.103	0.236	10.6	20.6	
	N	18 of 18	18 of 18	18 of 18	18 of 18	6 of 6
	Mean	0.101	0.201	10.0	20.8	38.4
	Standard Dev.	0.0129	0.0237	0.727	0.646	0.858
	%CV	**12.9**	**11.8**	**7.2**	**3.1**	**2.2**
	Accuracy	**100.6**	**93.4**	**100.5**	**103.9**	**96**

PZA

The overall accuracy and precision of calibration standards and QCs for PZA are summarised in Tables 6.9 and 6.10.

Table 6.9: Overall Summary of Calibration Standard Accuracy and Precision: Validation 1-3: PZA

	Sample ID	**STD 8 - LLOQ**	**STD 7**	**STD 6**	**STD 5**	**STD 4**	**STD 3**	**STD 2**	**STD 1**
	Nominal Conc.	**0.320**	**0.640**	**1.71**	**4.27**	**10.7**	**26.7**	**53.3**	**80**
		µg/ml	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**
Validation 1	**Data Point #1**	0.321	0.647	1.53	4.24	10.6	*	59.6	72.7
	Data Point #2	0.323	0.649	1.698	4.00	11.7	29.0	*	76.0
Validation 2	**Data Point #1**	0.323	0.651	1.592	4.18	10.6	28.3	50.7	*
	Data Point #2	0.306	0.704	1.613	4.24	10.8	27.9	58.1	72.2
Validation 3	**Data Point #1**	0.339	0.646	1.563	4.31	10.2	25.4	56.8	72.6
	Data Point #2	0.293	0.692	1.633	4.37	11.0	28.5	59.7	72.7
	N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	5 of 6	5 of 6	5 of 6
	Mean	0.317	0.665	1.604	4.22	10.8	27.8	57.0	73.2
	Standard Dev.	0.016	0.0263	0.0597	0.130	0.497	1.39	3.71	1.58
	%CV	**5**	**4**	**3.7**	**3.1**	**4.6**	**5.0**	**6.5**	**2.2**
	Accuracy	**99.2**	**103.9**	**93.8**	**98.9**	**101.1**	**104.2**	**106.9**	**91.5**

*Failed to meet acceptance criteria

Table 6.10: Overall Quality Control Accuracy and Precision Estimation: PZA

	Sample ID	**LLOQ**	**QCL**	**QCM**	**QCH**	**QC DIL**
	Nominal Conc.	**0.320**	**0.688**	**32.0**	**64.0**	**128**
		(µg/ml)	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**
	Replicates	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**
Validation 1	**Data Point #1**	0.298	0.620	25.1	64.7	121
	Data Point #2	0.270	0.641	27.3	61.3	122
	Data Point #3	0.281	0.623	28.6	64.9	120
	Data Point #4	0.301	0.592	28.1	63.3	120
	Data Point #5	0.290	0.617	29.6	60.2	123
	Data Point #6	0.297	0.652	28.4	67.7	117
Validation 2	**Data Point #1**	0.317	0.652	28.7	63.4	
	Data Point #2	0.308	0.624	29.2	64.4	
	Data Point #3	0.356	0.646	29.0	65.6	
	Data Point #4	0.343	0.674	30.1	65.6	
	Data Point #5	0.340	0.635	29.9	66.3	
	Data Point #6	0.303	0.661	30.2	68.7	
Validation 3	**Data Point #1**	0.266	0.592	30.1	66.0	
	Data Point #2	0.295	0.563	28.4	63.5	
	Data Point #3	0.272	0.611	31.4	65.2	
	Data Point #4	0.316	0.628	30.2	71.4	
	Data Point #5	0.292	0.640	30.7	66.5	
	Data Point #6	0.282	0.617	32.4	68.5	
	N	18 of 18	18 of 18	18 of 18	18 of 18	6 of 6
	Mean	0.301	0.627	29.3	65.4	120
	Standard Dev.	0.0252	0.027	1.62	2.68	2.16
	%CV	**8.4**	**4.3**	**5.5**	**4.1**	**1.8**
	Accuracy	**94.2**	**91.2**	**91.6**	**102.2**	**94.0**

RIF

The overall accuracy and precision of calibration standards and QCs for RIF are summarised in Tables 6.11 and 6.12.

Table 6.11: Overall Summary of Calibration Standard Accuracy and Precision: Validation 1-3: RIF

	Sample ID	**STD 8 - LLOQ**	**STD 7**	**STD 6**	**STD 5**	**STD 4**	**STD 3**	**STD 2**	**STD 1**
	Nominal Conc.	**0.120**	**0.240**	**0.640**	**1.60**	**4.00**	**10.0**	**20.0**	**30.0**
		µg/ml	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**	**µg/ml**
Validation 1	**Data Point #1**	0.119	0.265	0.570	1.70	3.83	9.95	20.6	30.3
	Data Point #2	0.117	0.249	0.608	1.62	4.10	9.95	19.8	29.5
Validation 2	**Data Point #1**	0.122	0.254	0.589	1.57	4.12	10.0	19.5	29.8
	Data Point #2	0.117	0.251	0.617	1.61	3.95	10.3	19.9	30.5
Validation 3	**Data Point #1**	0.134	0.250	0.586	1.61	4.04	9.62	19.7	29.5
	Data Point #2	0.109	0.250	0.611	1.63	3.96	10.1	20.9	30.1
	N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
	Mean	0.120	0.253	0.597	1.62	4.00	9.99	20.1	30.0
	Standard Dev.	0.00832	0.00621	0.0179	0.0418	0.108	0.216	0.538	0.433
	%CV	**6.9**	**2.5**	**3.0**	**2.6**	**2.7**	**2.2**	**2.7**	**1.4**
	Accuracy	**99.7**	**105.5**	**93.3**	**101.5**	**100.0**	**99.9**	**100.3**	**99.9**

Table 6.12: Overall Quality Control Accuracy and Precision Estimation: RIF

	Sample ID	**LLOQ**	**QCL**	**QCM**	**QCH**	**QC DIL**
	Nominal Conc.	**0.120**	**0.258**	**12.0**	**24.0**	**48.0**
		(µg/ml)	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**
	Replicates	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**	**Observed Conc.**
Validation 1	**Data Point #1**	0.124	0.246	10.4	24.5	45.1
	Data Point #2	0.114	0.241	10.8	24.1	45.4
	Data Point #3	0.119	0.245	11.0	23.3	44.1
	Data Point #4	0.128	0.248	11.1	23.5	44.0
	Data Point #5	0.127	0.254	10.9	23.4	45.1
	Data Point #6	0.124	0.268	11.0	23.4	42.2
Validation 2	**Data Point #1**	0.124	0.254	12.1	25.7	
	Data Point #2	0.126	0.263	12.9	26.6	
	Data Point #3	0.129	0.260	12.4	26.9	
	Data Point #4	0.121	0.239	12.4	27.1	
	Data Point #5	0.129	0.283	12.5	26.8	
	Data Point #6	0.134	0.248	12.4	25.8	
Validation 3	**Data Point #1**	0.126	0.253	12.2	26.0	
	Data Point #2	0.118	0.246	12.5	26.6	
	Data Point #3	0.114	0.240	12.7	25.8	
	Data Point #4	0.120	0.246	12.3	24.9	
	Data Point #5	0.124	0.260	12.3	24.7	
	Data Point #6	0.119	0.242	12.1	24.3	
	N	18 of 18	18 of 18	18 of 18	18 of 18	6 of 6
	Mean	0.123	0.252	11.9	25.2	44.3
	Standard Dev.	0.00544	0.0112	0.784	1.34	1.21
	%CV	**4.4**	**4.4**	**6.6**	**5.3**	**2.7**
	Accuracy	**102.7**	**97.7**	**99.0**	**104.9**	**92.3**

All intra- and inter-day accuracies and precision were below 15%, hence the method is accurate and precise for all analytes.

6.2. Specificity

Specificity is the ability of the assay to measure only the intended analyte(s). Representative chromatograms of STD 1 for each of the six analytes separately as well as a chromatogram showing all the analytes together are presented in Figure 6.1 to 6.6.

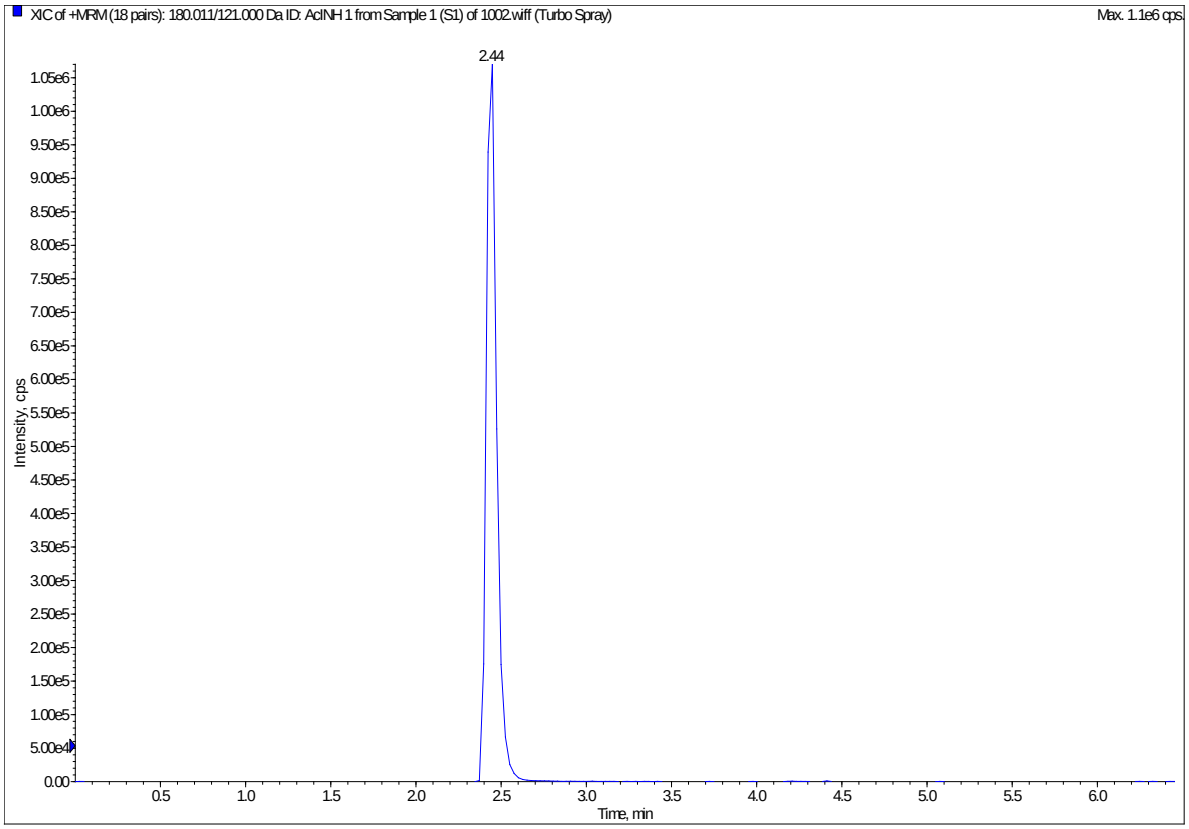

Figure 6.1: Representative chromatogram of STD 1: AcINH

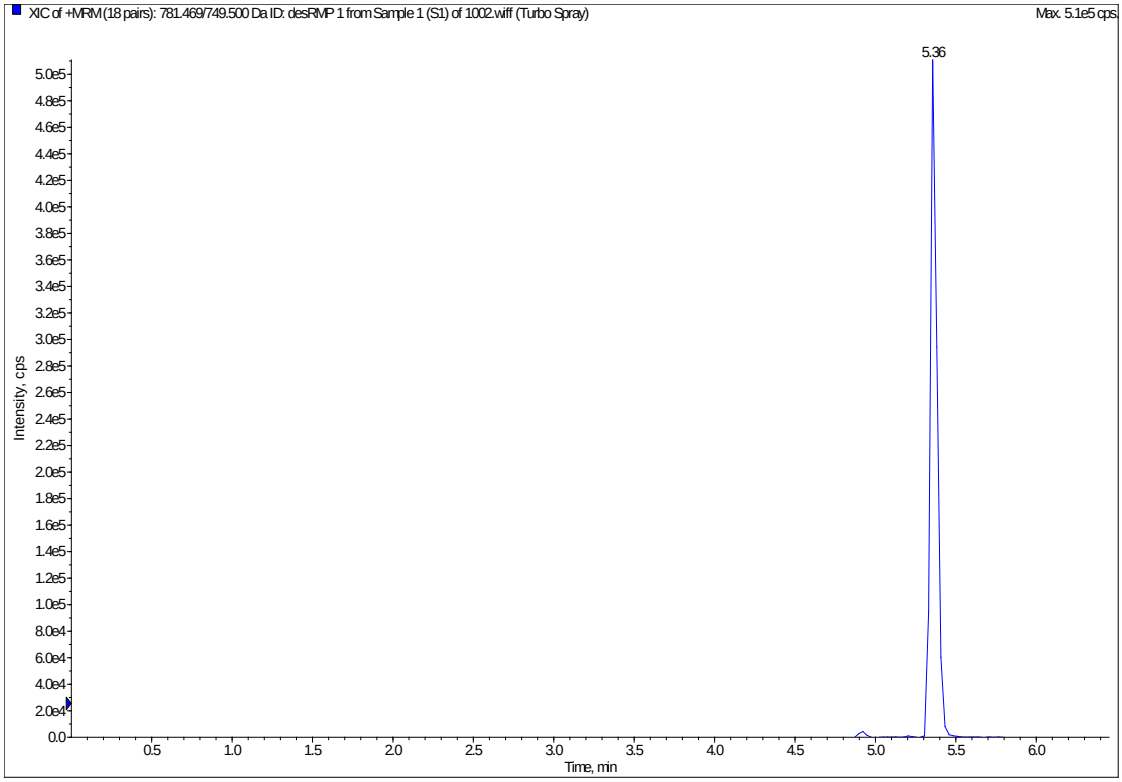

Figure 6.2: Representative chromatogram of STD 1: desRIF

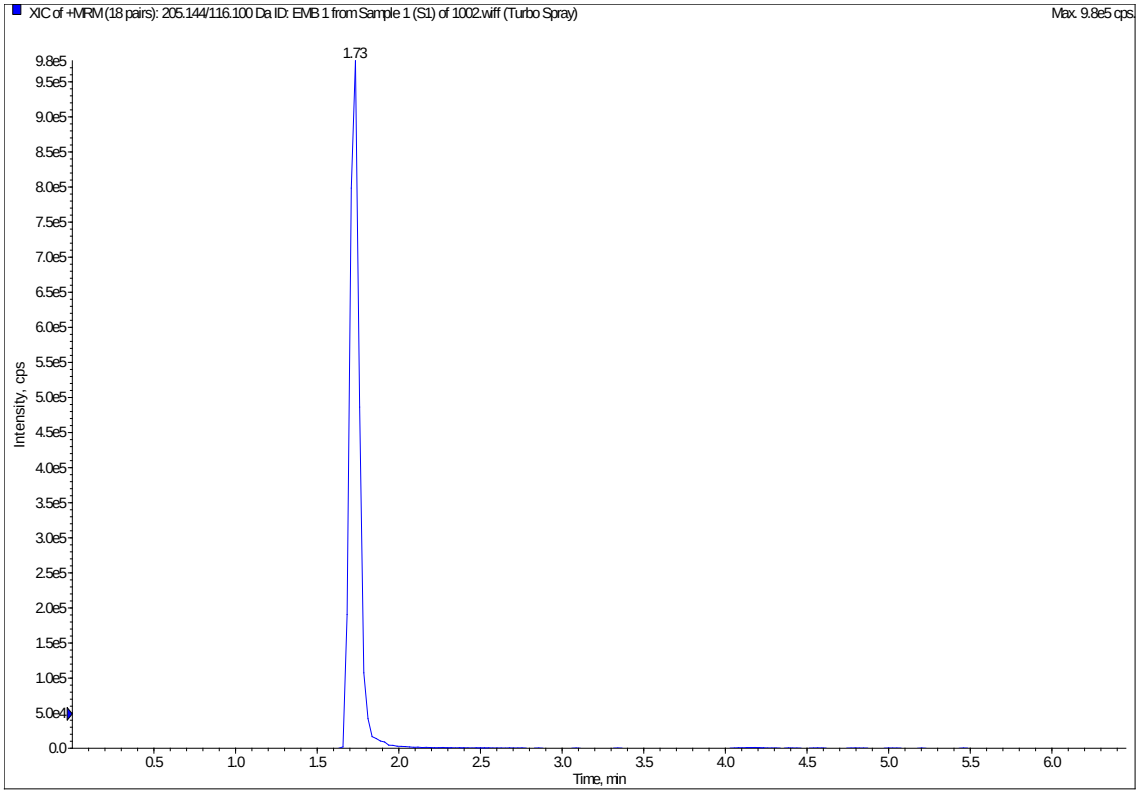

Figure 6.3: Representative chromatogram of STD 1: EMB

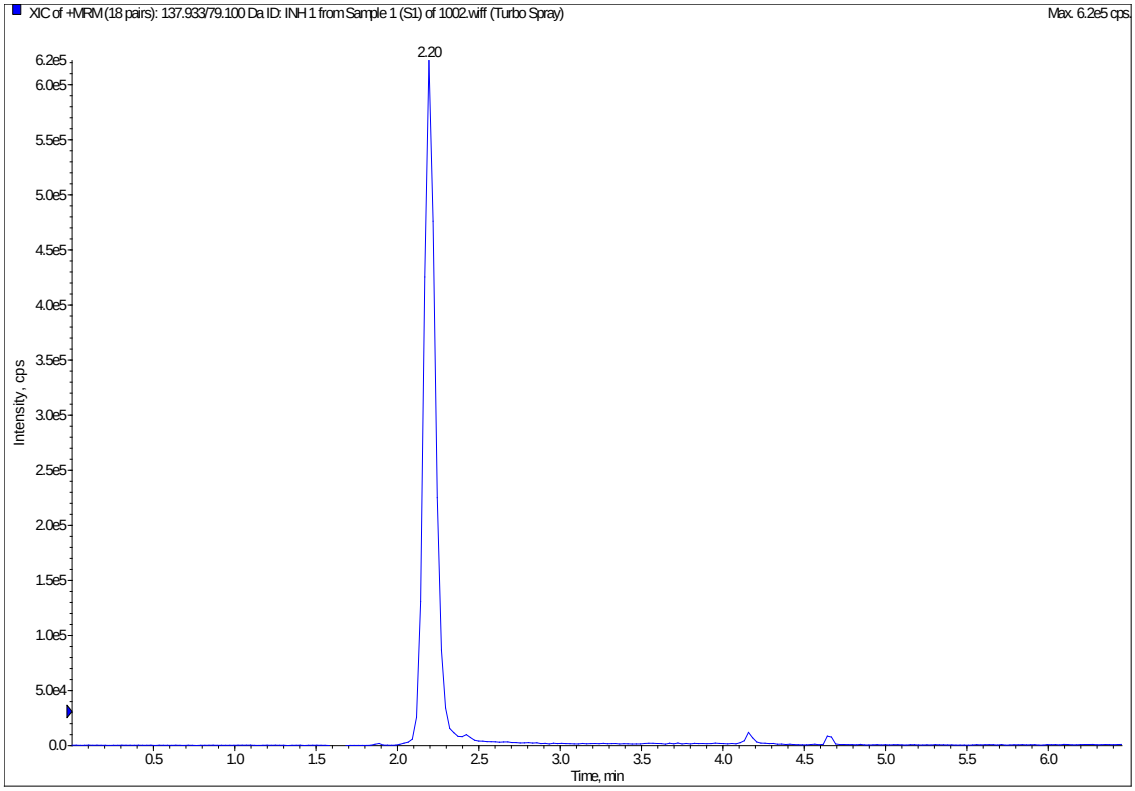

Figure 6.4: Representative chromatogram of STD 1: INH

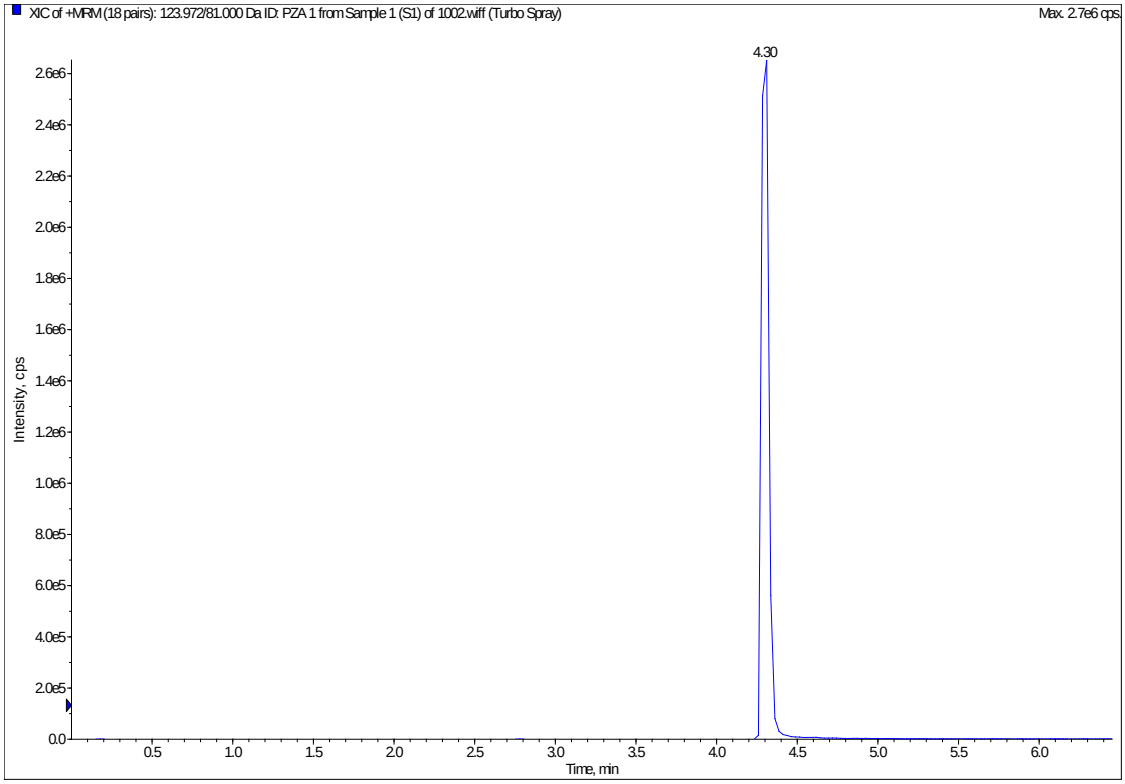

Figure 6.5: Representative chromatogram of STD 1: PZA

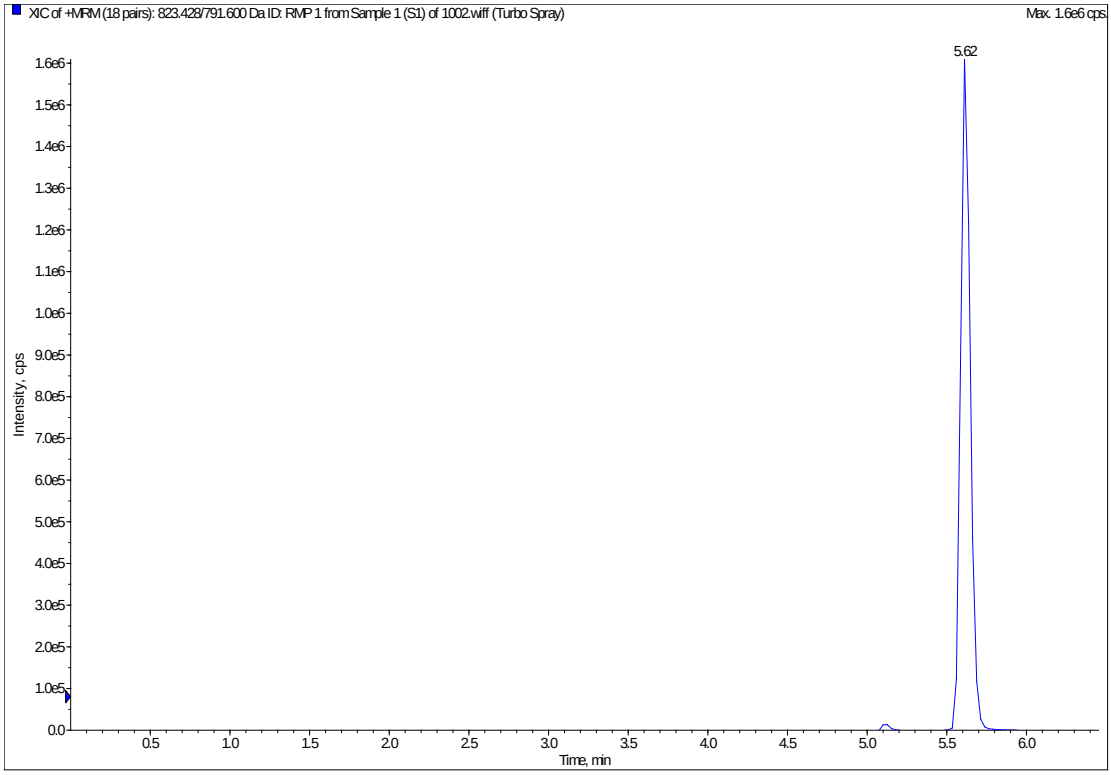

Figure 6.6: Representative chromatogram of STD 1: RIF

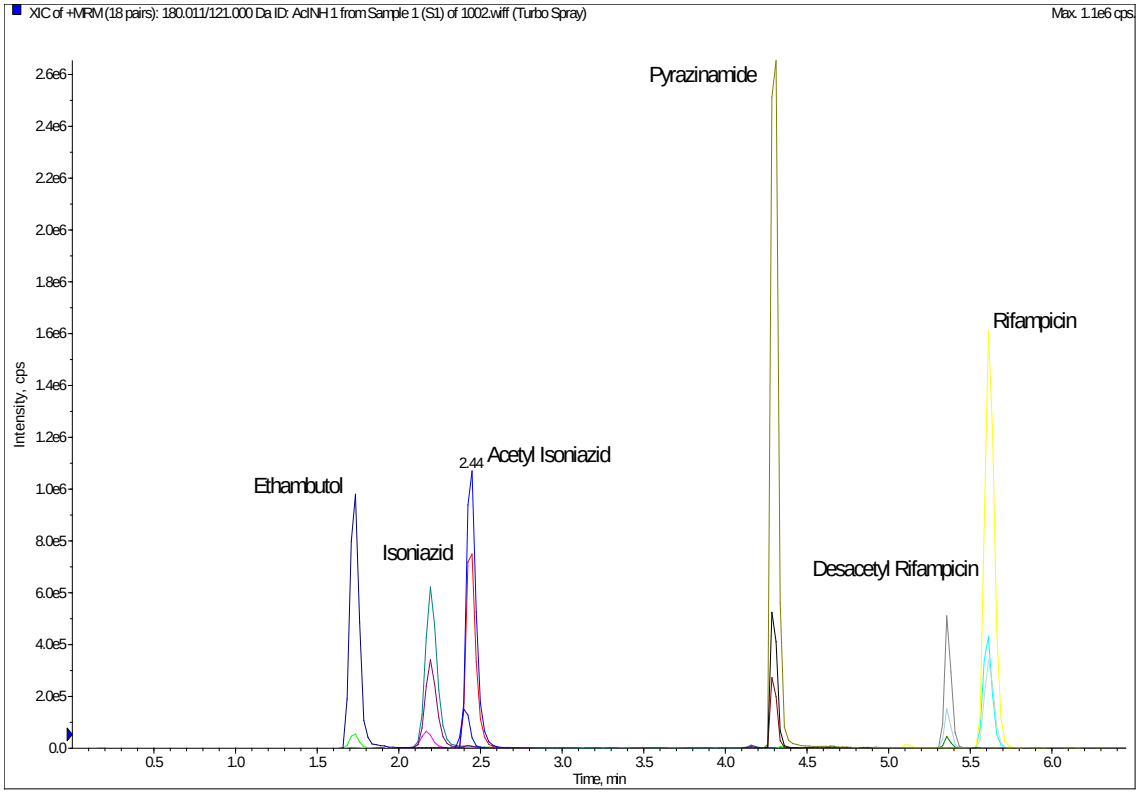

Figure 6.7: Representative chromatogram of STD 1 for all six analytes: Acetyl Isoniazid, 25-Desacetyl Rifampicin, Ethambutol, Isoniazid, Pyrazinamide and Rifampicin

6.3. Sensitivity

Six different lots of matrix were prepared at the LLOQ concentration and extracted, to determine the average signal:noise (S/N) ratio at LLOQ. Representative chromatograms for each analyte are presented in Figures 6.8 to 6.13.

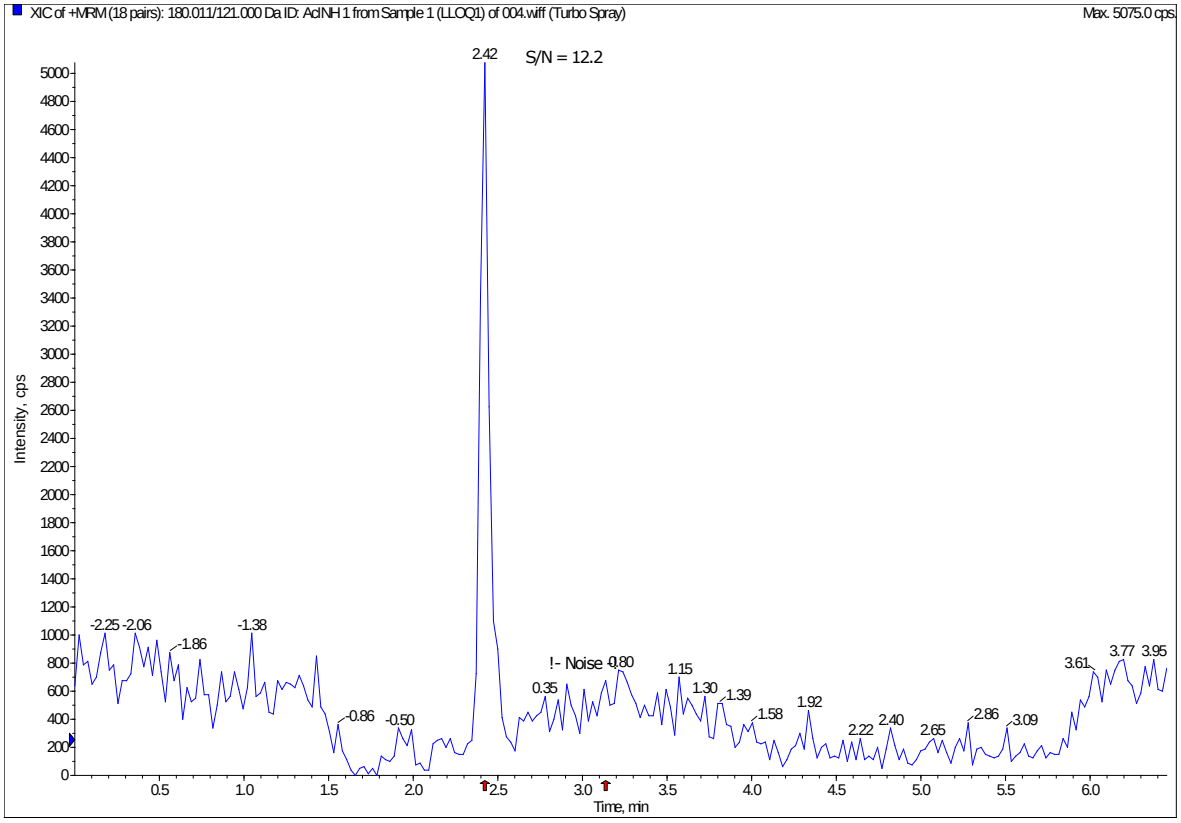

Figure 6.8: AcINH Raw chromatogram: Sample 1 spiked at LLOQ

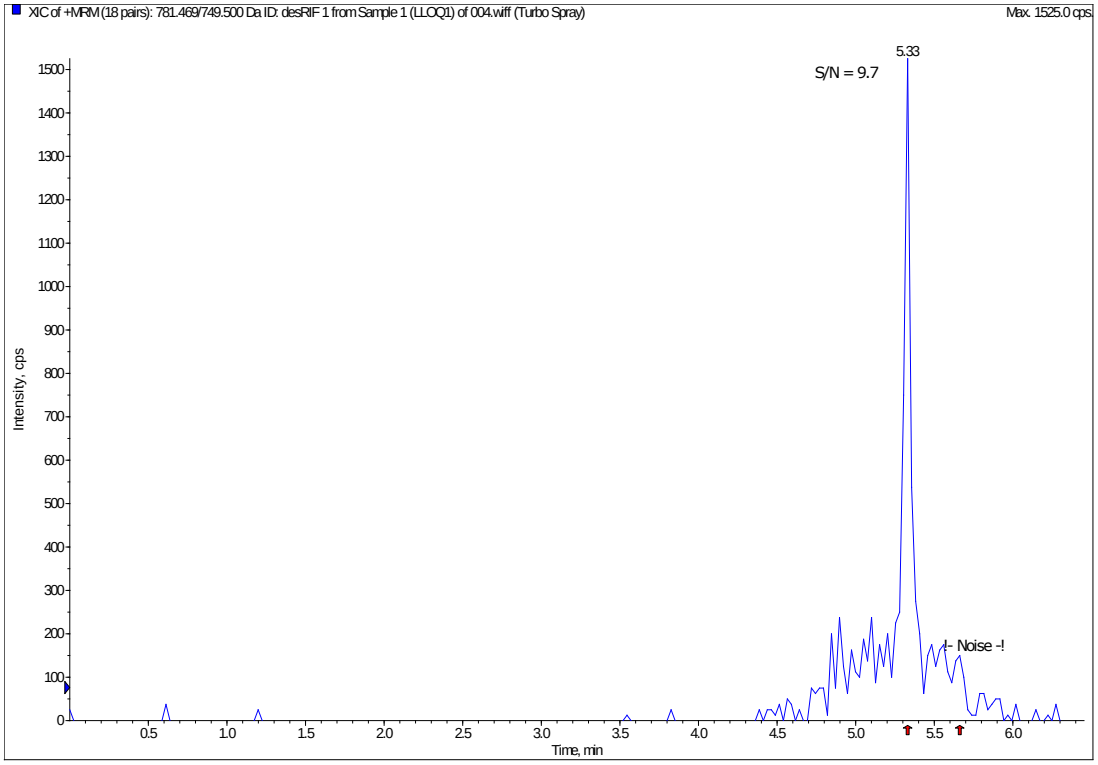

Figure 6.9: desRIF Raw chromatogram: Sample 1 spiked at LLOQ

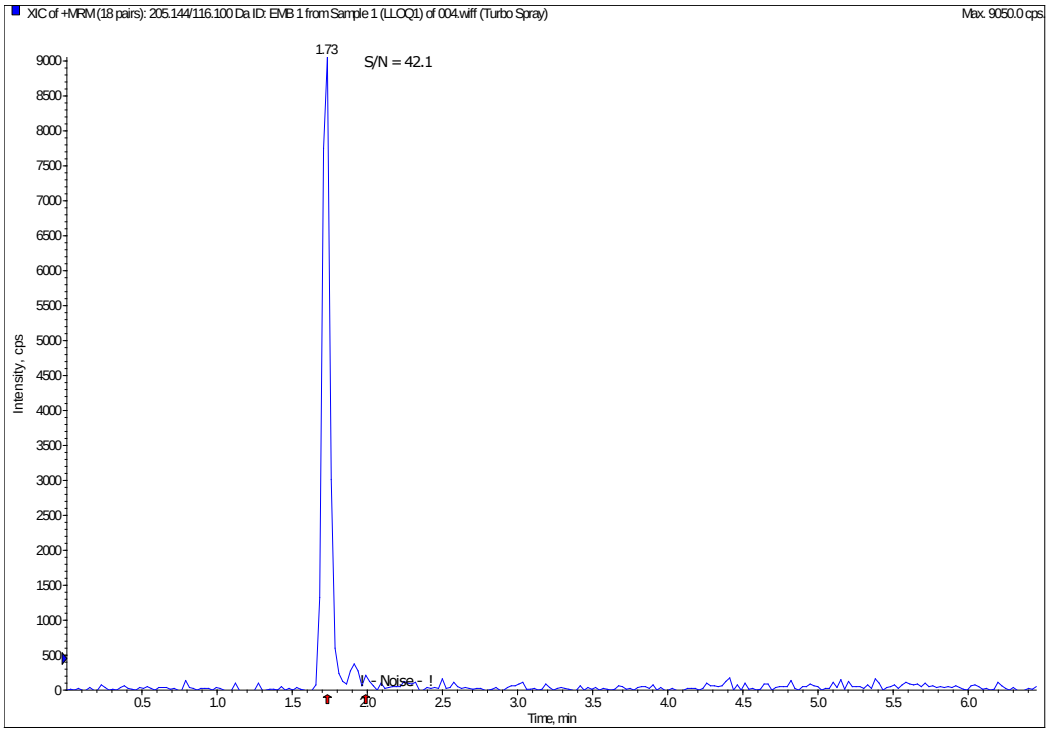

Figure 6.10: EMB Raw chromatogram: Sample 1 spiked at LLOQ

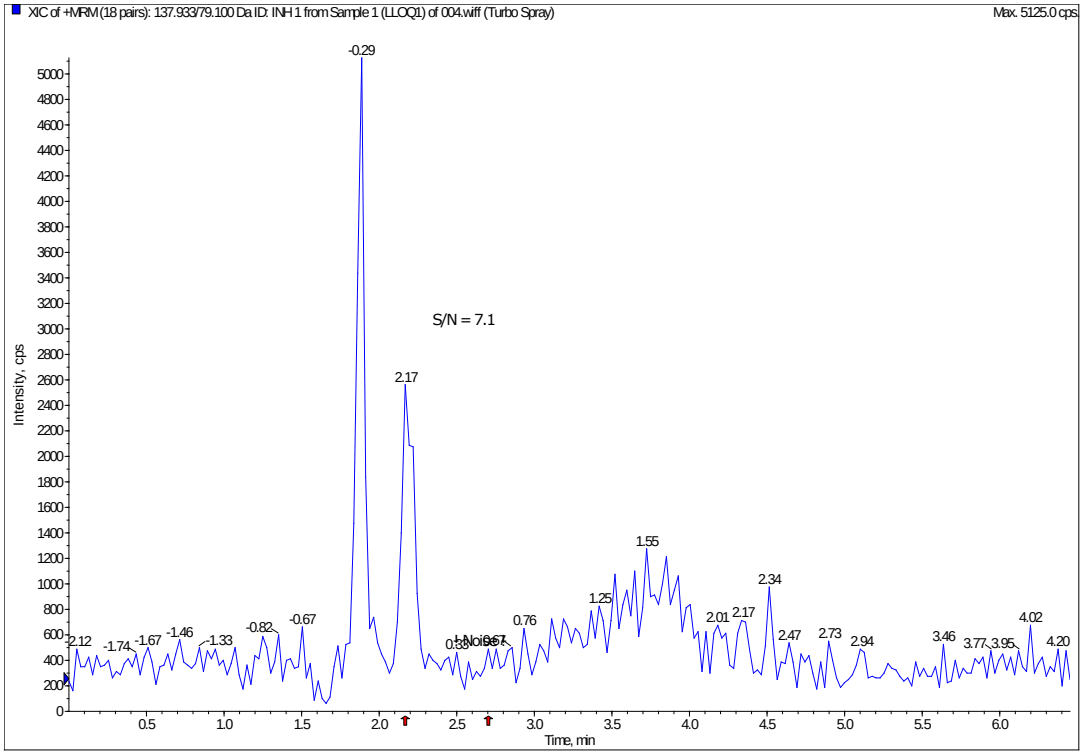

Figure 6.11: INH Raw chromatogram: Sample 1 spiked at LLOQ

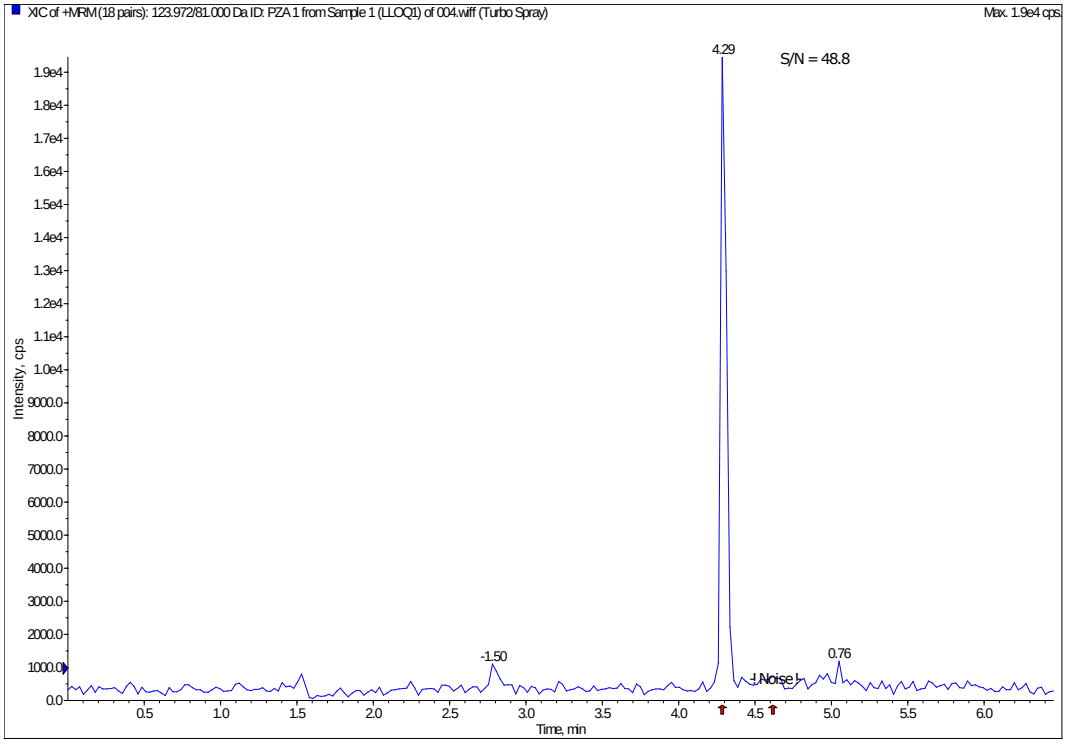

Figure 6.12: PZA Raw chromatogram: Sample 1 spiked at LLOQ

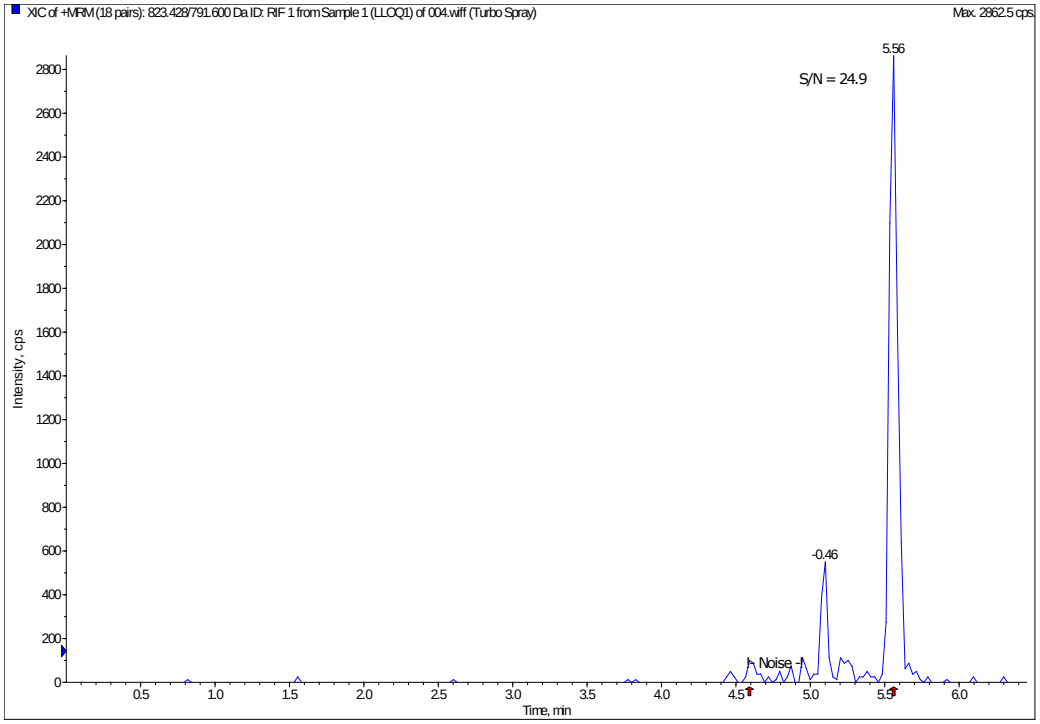

Figure 6.13: RIF Raw chromatogram: Sample 1 spiked at LLOQ

Average S/N ratios of 14.2, 8.43, 44.2, 7.52, 40.2 and 28.5 for AcINH, desRIF, EMB, INH, PZA and RIF respectively, calculated from 6 LLOQ samples were obtained. Average S/N ratio must be above 5 to meet the acceptance criteria for sensitivity, therefore the method was sensitive enough for all analytes.

6.4. Stability

Various stability experiments were performed to show that all the necessary precautions were taken to ensure that the analyte concentrations were not affected by the assay procedure or associated conditions.

6.4.1. Stock solution accuracy and stability

6.4.1.1. Stock solution accuracy

Two stock solutions (SS1 and SS2) of each of the analytes AcINH, desRIF, EMB, INH, PZA and RIF were prepared in methanol. Accuracy was determined by comparing the stock solutions prepared by two different analysts on the same day using ultraviolet-visible spectrophotometry for all analytes except EMB. The absorbance of each stock solution, prepared in triplicate in methanol at 30.0 (AcINH, desRIF, INH, RIF) or 50 µg/ml (PZA), were measured at specific wavelengths and compared. The stock solution accuracy of two stock solutions (SS1 and SS2) of EMB, prepared by two different analysts on the same day, was determined by LC/MS/MS analysis. Analyte solutions were prepared in triplicate in methanol at 3.0 µg/ml. The results are presented in Tables 6.13 – 6.18 below.

Table 6.13: Stock solution accuracy of AcINH solutions measured at a wavelength of 204 nm

	Absorbance at λ = 204 nm (AU)	
	AJ_22Jun2018	**MM_22Jun2018**
Absorbance A	1.48	1.41
Absorbance B	1.48	1.41
Absorbance C	1.48	1.41
Average	1.48	1.41
STDEV	0.00414	0.00372
% CV	0.28	0.26
% Difference		-4.7

Table 6.14: Stock solution accuracy of desRIF solutions measured at a wavelength of 237 nm

	Absorbance at λ = 237 nm (AU)	
	AJ_22Jun2018	**WvD_18Jun2018**
Absorbance A	1.24	1.23
Absorbance B	1.24	1.23
Absorbance C	1.24	1.23
Average	1.24	1.23
STDEV	0.00175	0.000513
% CV	0.14	0.04
% Difference		-0.8

Table 6.15: Stock solution accuracy of EMB solutions

Reference

AJ_22Jun2018	**Analyte Peak area**	**ISTD peak area**	**Peak Area Ratio**
Injection 1	2310000	6600000	0.350
Injection 2	2170000	6210000	0.349
Injection 3	1740000	4950000	0.352
Injection 4	2130000	6290000	0.339
Injection 5	1100000	3100000	0.355
Injection 6	1800000	5220000	0.345
Average	1875000	5395000	0.348
STDEV	439488	1297147	0.01
% CV	23.4	24.0	1.6

Test

MM_22Jun2018	**Analyte Peak area**	**ISTD peak area**	**Peak Area Ratio**
Injection 1	2140000	6030000	0.355
Injection 2	2280000	6080000	0.375
Injection 3	1340000	3720000	0.360
Injection 4	1350000	3800000	0.355
Injection 5	1150000	3180000	0.362
Injection 6	1740000	4780000	0.364
Average	1666667	4598333	0.362
STDEV	464658	1240845	0.007
% CV	27.9	27.0	2.0
% Difference			**3.9**

Table 6.16: Stock solution accuracy of INH solutions measured at a wavelength of 204 nm

	Absorbance at λ = 204 nm (AU)	
	AJ_22Jun2018	**MM_22Jun2018**
Absorbance A	1.72	1.65
Absorbance B	1.69	1.67
Absorbance C	1.70	1.65
Average	1.70	1.66
STDEV	0.012	0.01
% CV	0.7	0.6
% Difference		-2.7

Table 6.17: Stock solution accuracy of PZA solutions measured at a wavelength of 211 nm

	Absorbance at λ = 211 nm (AU)	
	AJ_22Jun2018	**MM_22Jun2018**
Absorbance A	2.78	2.91
Absorbance B	2.78	2.87
Absorbance C	2.78	2.91
Average	2.78	2.90
STDEV	0.00355	0.0184
% CV	0.1	0.6
% Difference		4.1

Table 6.18: Stock solution accuracy of RIF solutions measured at a wavelength of 237 nm

	Absorbance at λ = 237 nm (AU)	
	AJ_22Jun2018	**MM_22Jun2018**
Absorbance A	1.24	1.22
Absorbance B	1.24	1.22
Absorbance C	1.24	1.22
Average	1.24	1.22
STDEV	0.00182	0.000666
% CV	0.2	0.1
% Difference		-1.6

6.4.1.2. Stock solution stability

Stock solution stability was not tested in this validation because all analyte stock solutions have been shown to be stable at -80^{0}C in previous validations for the single drug assays (90-93).

6.4.2. Working solution stability

Working stock solutions, WS11 and WS15, containing AcINH, desRIF, EMB, INH, PZA and RIF were prepared in methanol. Aliquots of the working solutions were kept at room temperature and at ~4°C and frozen at ~-20°C and ~-80°C for 24 hours. The stability of the working solutions under these conditions for 24 hours was determined by comparing freshly prepared working solutions against the stored solutions. The results are shown in Tables 6.19 - 6.24 below.

Table 6.19: AcINH stability in high and low concentration working solutions at room temperature, ~4°C, ~-20°C and ~-80°C for 24 hours

	High Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	3.34	3.31	3.46	3.57	3.22
Peak area ratio 2	3.20	3.32	3.53	3.59	3.29
Peak area ratio 3	2.47	3.55	3.42	2.58	3.28
Peak area ratio 4	2.54	3.59	3.43	2.52	3.30
Peak area ratio 5	3.52	3.40	3.65	3.70	3.32
Peak area ratio 6	3.42	3.50	3.71	3.60	3.27
Average	3.08	3.45	3.53	3.26	3.28
STDEV	0.459	0.119	0.121	0.552	0.0341
% CV	14.9	3.5	3.4	16.9	1.0
% Difference		11.8	14.7	5.8	6.4
	Low Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	0.0215	0.0185	0.0224	0.0218	0.0189
Peak area ratio 2	0.0233	0.0221	0.0237	0.0191	0.0209
Peak area ratio 3	0.0229	0.0199	0.0269	0.0226	0.0208
Peak area ratio 4	0.0207	0.0204	0.0275	0.0196	0.0206
Peak area ratio 5	0.0229	0.0217	0.0238	0.0216	0.0163
Peak area ratio 6	0.0235	0.0217	0.0242	0.0206	0.0191
Average	0.0225	0.0207	0.0248	0.0209	0.0194
STDEV	0.00111	0.00138	0.002	0.00136	0.00177
% CV	5.0	6.7	8.1	6.5	9.1
% Difference		-7.8	10.2	-7.0	-13.5

Table 6.20: desRIF stability in high and low concentration working solutions at room temperature, ~4°C, ~-20°C and ~-80°C for 24 hours

	High Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	6.36	6.04	6.86	6.90	6.09
Peak area ratio 2	6.57	6.13	7.35	6.79	5.99
Peak area ratio 3	4.68	6.37	6.77	4.98	5.88
Peak area ratio 4	5.24	6.20	6.33	4.87	6.91
Peak area ratio 5	6.52	6.49	6.34	7.28	5.97
Peak area ratio 6	7.00	6.52	6.58	6.85	6.36
Average	6.06	6.29	6.71	6.28	6.20
STDEV	0.897	0.198	0.383	1.06	0.385
% CV	14.8	3.1	5.7	16.9	6.2
% Difference		3.8	10.6	3.6	2.3
	Low Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	0.0437	0.0405	0.0459	0.0424	0.0580
Peak area ratio 2	0.045	0.0418	0.0482	0.0509	0.0479
Peak area ratio 3	0.0516	0.0439	0.0604	0.0456	0.0432
Peak area ratio 4	0.0455	0.0469	0.0630	0.0530	0.0526
Peak area ratio 5	0.0440	0.0448	0.0497	0.0505	0.0490
Peak area ratio 6	0.0538	0.0568	0.0557	0.0441	0.0432
Average	0.0473	0.0458	0.0538	0.0478	0.0490
STDEV	0.00432	0.00585	0.00696	0.00428	0.0057
% CV	9.1	12.8	12.9	9.0	11.6
% Difference		-3.1	13.9	1.0	3.6

Table 6.21: EMB Stability in high and low concentration working solutions at room temperature, ~4°C, ~-20°C and ~-80°C for 24 hours

	High Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	6.99	7.03	7.49	7.77	7.97
Peak area ratio 2	7.30	7.22	7.31	7.54	7.76
Peak area ratio 3	[5.30]	7.57	7.10	[5.50]	7.62
Peak area ratio 4	[5.14]	7.80	6.96	5.75	7.66
Peak area ratio 5	7.44	7.65	7.68	7.86	7.44
Peak area ratio 6	7.41	7.51	7.43	7.82	8.16
Average	7.29	7.46	7.33	7.35	7.77
STDEV	0.206	0.286	0.264	0.9	0.259
% CV	2.8	3.8	3.6	12.3	3.3
% Difference		2.4	0.6	0.9	6.6
[] = statistical Outlier					
	Low Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	0.0421	0.0452	0.0454	0.0378	0.0456
Peak area ratio 2	0.0430	0.0468	0.0455	0.0415	0.0450
Peak area ratio 3	0.0452	0.0428	0.0552	0.0477	0.0412
Peak area ratio 4	0.0421	0.0411	0.0550	0.0499	0.0420
Peak area ratio 5	0.0456	0.0446	0.0476	0.0463	0.0461
Peak area ratio 6	0.0491	0.0389	0.0461	0.0442	0.0427
Average	0.0445	0.0432	0.0491	0.0446	0.0438
STDEV	0.00271	0.0029	0.00469	0.0044	0.00206
% CV	6.1	6.7	9.5	9.9	4.7
% Difference		-2.9	10.4	0.1	-1.7

Table 6.22: INH Stability in high and low concentration working solutions at room temperature, ~4°C, ~-20°C and ~-80°C for 24 hours

	High Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	5.52	5.43	5.81	6.38	5.63
Peak area ratio 2	5.46	5.74	5.77	6.19	5.69
Peak area ratio 3	4.47	5.83	5.82	4.36	5.47
Peak area ratio 4	4.45	6.45	5.72	4.73	5.66
Peak area ratio 5	6.00	5.56	5.92	6.57	5.65
Peak area ratio 6	5.91	6.67	6.06	6.37	5.77
Average	5.30	5.95	5.85	5.77	5.65
STDEV	0.685	0.5	0.122	0.961	0.099
% CV	12.9	8.4	2.1	16.7	1.7
% Difference		12.2	10.3	8.8	6.5
	Low Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	0.170	0.162	0.161	0.181	0.160
Peak area ratio 2	0.158	0.163	0.173	0.164	0.160
Peak area ratio 3	0.174	0.174	0.175	0.163	0.159
Peak area ratio 4	0.168	0.168	0.174	0.173	0.175
Peak area ratio 5	0.159	0.158	0.180	0.161	0.177
Peak area ratio 6	0.162	0.157	0.161	0.167	0.175
Average	0.165	0.164	0.171	0.168	0.168
STDEV	0.00646	0.00641	0.00787	0.00755	0.0088
% CV	3.9	3.9	4.6	4.5	5.2
% Difference		-0.9	3.3	1.8	1.5

Table 6.23: PZA Stability in high and low concentration working solutions at room temperature, ~4°C, ~-20°C and ~-80°C for 24 hours

	High Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	5.68	5.70	5.76	5.90	5.54
Peak area ratio 2	5.69	5.67	5.82	5.98	5.48
Peak area ratio 3	4.55	6.16	5.63	4.56	5.58
Peak area ratio 4	4.61	6.09	5.86	4.51	5.31
Peak area ratio 5	6.07	5.97	5.82	5.88	5.34
Peak area ratio 6	5.88	5.97	5.91	5.90	5.53
Average	5.41	5.93	5.80	5.46	5.46
STDEV	0.661	0.201	0.097	0.714	0.112
% CV	12.2	3.4	1.7	13.1	2.1
% Difference		9.5	7.1	0.8	0.9
	Low Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	0.0437	0.0418	0.0464	0.0403	0.0448
Peak area ratio 2	0.0418	0.0430	0.0449	0.0403	0.0412
Peak area ratio 3	0.0399	0.0412	0.0523	0.0442	0.0391
Peak area ratio 4	0.0419	0.0402	0.0563	0.0447	0.0406
Peak area ratio 5	0.0434	0.0413	0.0466	0.0428	0.0406
Peak area ratio 6	0.0446	0.0405	0.0470	0.0427	0.0391
Average	0.0426	0.0413	0.0489	0.0425	0.0409
STDEV	0.00169	0.001	0.00442	0.00187	0.0021
% CV	4.0	2.4	9.0	4.4	5.1
% Difference		-2.9	15	-0.1	-3.9

Table 6.24: RIF Stability in high and low concentration working solutions at room temperature, ~4°C, ~-20°C and ~-80°C for 24 hours

	High Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	5.52	5.43	5.81	6.38	5.63
Peak area ratio 2	5.46	5.74	5.77	6.19	5.69
Peak area ratio 3	4.47	5.83	5.82	4.36	5.47
Peak area ratio 4	4.45	6.45	5.72	4.73	5.66
Peak area ratio 5	6.00	5.56	5.92	6.57	5.65
Peak area ratio 6	5.91	6.67	6.06	6.37	5.77
Average	5.30	5.95	5.85	5.77	5.65
STDEV	0.685	0.5	0.122	0.961	0.099
% CV	12.9	8.4	2.1	16.7	1.7
% Difference		12.2	10.3	8.8	6.5
	Low Concentration				
	Reference	**Test (Room temp)**	**Control (~ 4 ºC)**	**Control (~- 20 ºC)**	**Control (~- 80 ºC)**
Peak area ratio 1	0.0105	0.0108	0.0102	0.00946	0.0109
Peak area ratio 2	0.00928	0.0105	0.0108	0.00969	0.00991
Peak area ratio 3	0.0109	0.00975	0.0138	0.0106	0.0101
Peak area ratio 4	0.00941	0.0101	0.0133	0.011	0.0108
Peak area ratio 5	0.0105	0.00934	0.0102	0.00978	0.00973
Peak area ratio 6	0.0107	0.0102	0.0107	0.0102	0.00970
Average	0.0102	0.0100	0.0115	0.0101	0.0102
STDEV	0.000691	0.000521	0.00161	0.000591	0.000532
% CV	6.8	5.2	14.0	5.8	5.2
% Difference		-1	12.6	-0.9	-0.2

Acceptance Criteria: A CV(%) higher than 15% of the measured values and a difference of more than 15% from the reference solution, could indicate instability in the working solution.

The results indicate that high and low concentrations of Ethambutol and Pyrazinamide are stable for 24 hours at room temperature, ~4°C, ~-20°C and ~-80°C. The CV(%) of high concentrations of AcINH, desRIF, INH and RIF are greater than 15% when stored at ~-20°C for 24 hours, suggesting that there could be instability in these working solutions under these conditions . However, the variability could be a result of outliers, hence further investigations are necessary.

6.4.3. Storage stability in matrix at -80°C

Stability of the analytes in plasma has been previously determined and was not repeated for this validation. Previous reports indicate that AcINH and INH are stable for up to 104 days at -80°C (92). DesRIF and RIF are stable for up to 392 and 483 days respectively at -80°C (91). EMB is stable for up to 564 days at -80°C (90) and PZA is stable for up to 300 days at -80°C (93).

6.4.4. Freeze and thaw stability

To ascertain freeze-thaw stability, low- and high-quality controls were frozen at ~-80ºC, and put through three consecutive freeze and thaw cycles. Sample aliquots were prepared and frozen for at least 24 hours prior to starting this experiment. Each cycle consisted of sufficient thawing time at room temperature followed by ~20 hours freezing time. These samples were analysed against a freshly prepared valid calibration curve and assessed for accuracy against the nominal QC concentration. The measured concentrations and calculated differences after three cycles for the two sets of quality controls are presented in Tables 6.25 – 6.30.

Table 6.25: Freeze and thaw stability of AcINH

	High Concentration		Low Concentration	
	Nominal QH (µg/ml)	**Observed FT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed FT QL (µg/ml)**
Sample 1	10	9.34	0.107	0.0910
Sample 2		9.18		0.0994
Sample 3		9.24		0.0902
Sample 4		9.40		0.0953
Sample 5		8.96		0.0839
Sample 6		9.41		0.0922
	Average	9.26	**Average**	0.0920
	STDEV	0.17	**STDEV**	0.0052
	% CV	1.8	**% CV**	5.7
	% Difference	-7.4	**% Difference**	-14.0

Table 6.26: Freeze and thaw stability of desRIF

	High Concentration		Low Concentration	
	Nominal QH (µg/ml)	**Observed FT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed FT QL (µg/ml)**
Sample 1	8.00	8.33	0.0860	0.0758
Sample 2		8.16		0.0644
Sample 3		7.34		0.0843
Sample 4		7.58		0.0898
Sample 5		7.55		0.0825
Sample 6		7.89		0.0852
	Average	7.81	**Average**	0.0803
	STDEV	0.385	**STDEV**	0.009
	% CV	4.9	**% CV**	11.2
	% Difference	-2.4	**% Difference**	-6.6

Table 6.27: Freeze and thaw stability of E**MB**

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed FT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed FT QL (µg/ml)**
Sample 1	4.00	3.98	0.0430	0.0400
Sample 2		3.99		0.0420
Sample 3		3.71		0.0435
Sample 4		3.94		0.0455
Sample 5		4.17		0.0357
Sample 6		4.07		0.0394
	Average	3.98	**Average**	0.0410
	STDEV	0.154	**STDEV**	0.0034
	% CV	3.9	**% CV**	8.4
	% Difference	-0.6	**% Difference**	-4.6

Table 6.28: Freeze and thaw stability of INH

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed FT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed FT QL (µg/ml)**
Sample 1	20.0	20.0	0.215	0.195
Sample 2		19.9		0.197
Sample 3		19.2		0.200
Sample 4		20.4		0.204
Sample 5		19.9		0.175
Sample 6		19.9		0.198
	Average	19.9	**Average**	0.195
	STDEV	0.387	**STDEV**	0.0102
	% CV	1.9	**% CV**	5.2
	% Difference	-0.6	**% Difference**	-9.4

Table 6.29: Freeze and thaw stability of PZA

	High Concentration		Low Concentration	
	Nominal QH (µg/ml)	**Observed FT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed FT QL (µg/ml)**
Sample 1	64.0	59.3	0.688	0.611
Sample 2		61.4		0.655
Sample 3		54.4		0.639
Sample 4		61.1		0.657
Sample 5		61.4		0.620
Sample 6		58.2		0.600
	Average	59.3	**Average**	0.630
	STDEV	2.73	**STDEV**	0.0236
	% CV	4.6	**% CV**	3.8
	% Difference	-7.3	**% Difference**	-8.4

Table 6.30: Freeze and thaw stability of RIF

	High Concentration		Low Concentration	
	Nominal QH (µg/ml)	**Observed FT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed FT QL (µg/ml)**
Sample 1	24.0	24.8	0.258	0.252
Sample 2		24.3		0.251
Sample 3		24.6		0.246
Sample 4		24.7		0.258
Sample 5		24.2		0.229
Sample 6		24.2		0.255
	Average	24.5	**Average**	0.249
	STDEV	0.266	**STDEV**	0.0104
	% CV	1.1	**% CV**	4.2
	% Difference	1.9	**% Difference**	-3.7

Acceptance criteria: A CV(%) and % Difference greater than 15% of the measured values could indicate freeze-thaw instability.

The CV(%) and % Difference for each of the six analytes were within 15%, indicating that the analytes are stable in plasma for at least three freeze-thaw cycles.

6.4.5. Bench top stability

To ascertain benchtop stability, low- and high-quality controls were frozen at ~-80ºC, and subsequently left on the bench, kept on ice, for approximately 4 hours (maximum anticipated time that future study samples will be left thawed until extracted). These samples were analysed against a valid fresh calibration curve. The measured concentrations and calculated accuracies for the quality controls are presented in Tables 6.31 - 6.36.

Table 6.31: Stability of AcINH for ~4 hours on bench on ice

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed BT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed BT QL (µg/ml)**
Sample 1	**10.0**	10.4	**0.107**	0.0953
Sample 2		10.5		0.0949
Sample 3		10.5		0.0940
Sample 4		10.8		0.0982
Sample 5		10.6		0.106
Sample 6		11.1		0.109
	Average	10.7	**Average**	0.100
	STDEV	0.3	**STDEV**	0.01
	% CV	2.4	**% CV**	6.4
	% Difference	6.5	**% Difference**	-6.9

Table 6.32: Stability of desRIF for ~4 hours on bench on ice

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed BT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed BT QL (µg/ml)**
Sample 1	**8.00**	8.06	**0.0860**	0.0863
Sample 2		8.30		0.0827
Sample 3		9.06		0.0949
Sample 4		7.85		0.0893
Sample 5		8.23		0.0836
Sample 6		7.30		0.0887
	Average	8.13	**Average**	0.0876
	STDEV	0.579	**STDEV**	0.004
	% CV	7.1	**% CV**	5.1
	% Difference	1.7	**% Difference**	1.8

Table 6.33: Stability of Ethambutol for ~4 hours on bench on ice

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed BT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed BT QL (µg/ml)**
Sample 1	**4.00**	4.06	**0.0430**	0.0415
Sample 2		4.50		0.0385
Sample 3		4.24		0.0383
Sample 4		4.43		0.0366
Sample 5		4.54		0.0384
Sample 6		4.31		0.0384
	Average	4.35	**Average**	0.0386
	STDEV	0.18	**STDEV**	0.002
	% CV	4.2	**% CV**	4.1
	% Difference	8.7	**% Difference**	-10.2

Table 6.34: Stability of Isoniazid for ~4 hours on bench on ice

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed BT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed BT QL (µg/ml)**
Sample 1	**20.0**	20.7	**0.215**	0.186
Sample 2		21.4		0.180
Sample 3		20.7		0.203
Sample 4		22.7		0.194
Sample 5		20.8		0.188
Sample 6		22.0		0.198
	Average	21.4	**Average**	0.192
	STDEV	0.823	**STDEV**	0.008
	% CV	3.8	**% CV**	4.4
	% Difference	6.9	**% Difference**	-10.9

Table 6.35: Stability of Pyrazinamide for ~4 hours on bench on ice

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed BT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed BT QL (µg/ml)**
Sample 1	**64.0**	66.8	**0.688**	0.600
Sample 2		71.3		0.558
Sample 3		71.2		0.622
Sample 4		69.0		0.637
Sample 5		70.7		0.600
Sample 6		71.4		0.622
	Average	70.1	**Average**	0.607
	STDEV	1.83	**STDEV**	0.028
	% CV	2.6	**% CV**	4.6
	% Difference	9.5	**% Difference**	-11.8

Table 6.36 The stability of Rifampicin for ~4 hours on bench on ice

	High Concentration		**Low Concentration**	
	Nominal QH (µg/ml)	**Observed BT QH (µg/ml)**	**Nominal QL (µg/ml)**	**Observed BT QL (µg/ml)**
Sample 1	**24.0**	25.4	**0.258**	0.247
Sample 2		25.8		0.246
Sample 3		26.1		0.233
Sample 4		26.4		0.236
Sample 5		26.1		0.245
Sample 6		26.7		0.227
	Average	26.1	**Average**	0.239
	STDEV	0.454	**STDEV**	0.008
	% CV	1.7	**% CV**	3.4
	% Difference	8.7	**% Difference**	-7.4

Acceptance criteria: A CV(%) and % Difference greater than 15% of the measured values could indicate on-bench instability.

The CV(%) and % Difference for all six analytes were within 15%. All analytes were therefore stable in plasma for at least 4 hours when kept on ice.

6.4.6. Reinjection reproducibility

Reinjection reproducibility is evaluated to determine if an analytical run can be reanalysed by reinjection in the case of instrument interruptions. Following the injection of a validation run (Validation 1), the extracted samples (in the 96-well plate) remain in the autosampler at the method-defined temperature for the following ~48 hours. The analytical run is reinjected in its entirety after ~48 hours. This will demonstrate reinjection reproducibility for ~48 hours as presented in Tables 6.37 – 6.48.

Table 6.37: Calibration Standards Accuracy and Precision – Validation 1 Reinjected after ~48 hours: AcINH

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	n
S8	0.0500	0.0519	0.001	1.6	103.9	2 of 2
S7	0.100	0.104	0.002	1.6	104.3	2 of 2
S6	0.270	0.243	N/A	N/A	90.0	1 of 2
S5	0.670	0.667	0.034	5.1	99.6	2 of 2
S4	1.67	1.61	0.123	7.6	96.6	2 of 2
S3	4.17	4.08	0.004	0.1	97.8	2 of 2
S2	8.33	8.72	0.18	2.1	104.7	2 of 2
S1	12.5	12.3	0.591	4.8	98.1	2 of 2

Table 6.38: Quality Control Accuracy and Precision – Validation 1 Reinjected after ~48 hours:

AcINH

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	n
LLOQ	0.0500	0.0471	0.002	3.8	94.2	6 of 6
QCL	0.107	0.0970	0.007	7.6	90.6	6 of 6
QCM	5.00	4.54	0.261	5.7	90.8	6 of 6
QCH	10.0	10.2	0.2	1.9	102.4	6 of 6

Table 6.39: Calibration Standards Accuracy and Precision – Validation 1 Reinjected after ~48 hours:

desRIF

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S8	0.0400	0.0406	0.006	15.2	101.4	2 of 2
S7	0.0800	0.0877	0.001	1.3	109.6	2 of 2
S6	0.213	0.190	0.008	4.4	89.4	2 of 2
S5	0.533	0.541	0.039	7.2	101.5	2 of 2
S4	1.33	1.28	0.2	15.7	96.2	2 of 2
S3	3.33	3.38	0.259	7.7	101.4	2 of 2
S2	6.67	6.81	0.318	4.7	102.0	2 of 2
S1	10.0	9.84	0.478	4.9	98.4	2 of 2

Table 6.40: Quality Control Accuracy and Precision – Validation 1 Reinjected after ~48 hours:

desRIF

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
LLOQ	0.0400	0.0360	0.004	10.5	89.9	6 of 6
QCL	0.0860	0.0783	0.004	5.1	91.1	6 of 6
QCM	4.00	3.66	0.333	9.1	91.5	6 of 6
QCH	8.00	8.23	0.48	5.9	102.8	6 of 6

Table 6.41: Calibration Standards Accuracy and Precision – Validation 1 Reinjected after ~48 hours:

EMB

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S8	0.0200	0.0221	0.001	3.5	110.6	2 of 2
S7	0.0400	0.0421	0.001	3.1	105.3	2 of 2
S6	0.107	0.0985	0.005	5.0	92.0	2 of 2
S5	0.267	0.254	0.007	2.8	95.0	2 of 2
S4	0.667	0.632	0.033	5.3	94.8	2 of 2
S3	1.67	1.65	0.005	0.3	99.0	2 of 2
S2	3.33	3.52	0.285	8.1	105.7	2 of 2
S1	5.00	4.88	0.155	3.2	97.6	2 of 2

Table 6.42: Quality Control Accuracy and Precision – Validation 1 Reinjected after ~48 hours: EMB

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
LLOQ	0.0200	0.0221	0.002	7.7	110.6	6 of 6
QCL	0.0430	0.0429	0.001	3.2	99.7	6 of 6
QCM	2.00	1.89	0.109	5.7	94.6	6 of 6
QCH	4.00	4.08	0.201	4.9	102.0	6 of 6

Table 6.43: Calibration Standards Accuracy and Precision – Validation 1 Reinjected after ~48 hours: INH

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S8	0.100	0.110	N/A	N/A	110.0	1 of 2
S7	0.200	0.221	0.006	2.6	110.3	2 of 2
S6	0.533	0.471	0.03	5.5	88.4	2 of 2
S5	1.33	1.30	0.024	1.9	98.1	2 of 2
S4	3.33	3.19	0.36	11.3	95.8	2 of 2
S3	8.33	8.44	0.361	4.3	101.3	2 of 2
S2	16.7	17.1	0.558	3.3	102.1	2 of 2
S1	25.0	24.7	1.03	4.2	98.9	2 of 2

Table 6.44: Quality Control Accuracy and Precision – Validation 1 Reinjected after ~48 hours: INH

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
LLOQ	0.100	0.107	0.006	5.9	106.8	6 of 6
QCL	0.215	0.190	0.017	9.1	88.2	5 of 6
QCM	10.0	8.90	0.558	6.3	89.0	6 of 6
QCH	20.0	19.8	0.358	1.8	99.2	6 of 6

Table 6.45: Calibration Standards Accuracy and Precision – Validation 1 Reinjected after ~48 hours: PZA

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S8	0.320	0.329	0.03	9.1	102.9	2 of 2
S7	0.640	0.666	0.024	3.7	104.1	2 of 2
S6	1.71	1.62	N/A	N/A	94.5	1 of 2
S5	4.27	4.31	0.098	2.3	101.0	2 of 2
S4	10.7	9.80	0.715	7.3	91.9	2 of 2
S3	26.7	26.7	0.583	2.2	99.9	2 of 2
S2	53.3	57.2	0.513	0.9	107.4	2 of 2
S1	80.0	72.4	N/A	N/A	90.5	1 of 2

Table 6.46: Quality Control Accuracy and Precision – Validation 1 Reinjected after ~48 hours: PZA

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
LLOQ	0.320	0.282	0.017	6.1	88.2	6 of 6
QCL	0.688	0.572	0.028	5.0	83.2	6 of 6
QCM	32.0	24.4	1.34	5.5	76.4	6 of 6
QCH	64.0	59.0	2.11	3.6	92.1	6 of 6

Table 6.47: Quality Control Accuracy and Precision – Validation 1 Reinjected after ~48 hours: RIF

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S8	0.120	0.122	0.003	2.7	101.7	2 of 2
S7	0.240	0.261	0.008	3.2	108.6	2 of 2
S6	0.640	0.585	0.025	4.3	91.4	2 of 2
S5	1.60	1.60	0.036	2.2	100.3	2 of 2
S4	4.00	3.87	0.242	6.2	96.6	2 of 2
S3	10.0	10.0	0.226	2.3	100.2	2 of 2
S2	20.0	20.5	0.15	0.7	102.6	2 of 2
S1	30.0	29.6	1.42	4.8	98.7	2 of 2

Table 6.48: Quality Control Accuracy and Precision – Validation 1 Reinjected after ~48 hours: RIF

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
LLOQ	0.120	0.125	0.005	4.0	104.0	6 of 6
QCL	0.258	0.255	0.008	3.1	98.9	6 of 6
QCM	12.0	10.7	0.242	2.3	89.2	6 of 6
QCH	24.0	24.2	0.278	1.1	100.8	6 of 6

Accuracy must be within 15% (between 85% - 115%) and precision <15%. The CV(%) and % Accuracy for the all of the analytes, except PZA, were within 15% indicating that an entire batch, stored at 8°C in the autosampler, may be re-injected after ~48 hours for analysing these analytes. Re-injection after 24 hours was done as well and the CV(%) and %Accuracy for all of the analytes, except Pyrazinamide, are within 15% and no differences in trends in the data recorded after 24 and 48 hour re-injections can be observed.

The data indicated that Pyrazinamide cannot be re-injected after ~48 hours when stored at 8°C in the autosampler as the % Accuracy of quality controls QCL and QCM does not fall within the acceptance criteria of 85% - 115%. The % Accuracy for the re-injection of quality control QCM after 24 hours also failed to meet the acceptance criteria.

6.4.7. Autosampler stability

To assess autosampler stability, the first validation run was reinjected twice; after approximately 26 (re-injection 1) and 50 (re-injection 2) hours at 8°C. The reinjected high- and

low-quality control peak area ratios were compared to those obtained during the first injection to estimate the absolute autosampler stability over ~50 hours. Results for autosampler stability at ~ 50 hour are presented in Tables 6.49 - 60

Table 6.49: Autosampler stability for extracted samples: High concentration for Acetyl Isoniazid

QC H (10.0 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	1840000	285000	6.46
Injection 2	1910000	311000	6.14
Injection 3	1990000	312000	6.38
Injection 4	2040000	312000	6.54
Injection 5	2090000	327000	6.39
Injection 6	2100000	326000	6.44
Average	1995000	312167	6.39
STDEV	103296	15171	0.135
% CV	5.2	4.9	2.1
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	3380000	540000	6.26
Injection 8	3210000	530000	6.06
Injection 9	3150000	512000	6.15
Injection 10	3120000	515000	6.06
Injection 11	3140000	506000	6.21
Injection 12	3030000	479000	6.33
Average	3171667	513667	6.18
STDEV	117544	21097	0.109
% CV	3.7	4.1	1.8
		% Difference after Re-injection 1	**-3.4**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	3640000	584000	6.23
Injection 14	3650000	600000	6.08
Injection 15	3580000	587000	6.10
Injection 16	3530000	571000	6.18
Injection 17	3300000	539000	6.12
Injection 18	3480000	546000	6.37
Average	3530000	571167	6.18
STDEV	129923	24145	0.109
% CV	3.7	4.2	1.8
		% Difference after Re-injection 2	**-3.3**

Table 6.50: Autosampler stability for extracted samples: Low concentration for Acetyl Isoniazid

QC L (0.107 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	17400	298000	0.0584
Injection 2	17200	304000	0.0566
Injection 3	20700	311000	0.0666
Injection 4	22700	333000	0.0682
Injection 5	20100	324000	0.0620
Injection 6	21000	325000	0.0646
Average	19850	315833	0.0627
STDEV	2157	13615	0.00458
% CV	10.9	4.3	7.3
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	36300	543000	0.0669
Injection 8	35200	547000	0.0644
Injection 9	33000	521000	0.0633
Injection 10	31900	491000	0.0650
Injection 11	31600	505000	0.0626
Injection 12	32300	474000	0.0681
Average	33383	513500	0.0650
STDEV	1924	28940	0.00211
% CV	5.8	5.6	3.3
		% Difference after Re-injection 1	**3.7**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	38800	610000	0.0636
Injection 14	34200	622000	0.0550
Injection 15	38700	557000	0.0695
Injection 16	35900	564000	0.0637
Injection 17	36300	555000	0.0654
Injection 18	34700	541000	0.0641
Average	36433	574833	0.0635
STDEV	1951	32969	0.00474
% CV	5.4	5.7	7.5
		% Difference after Re-injection 2	**1.3**

Table 6.51: Autosampler stability for extracted samples: High concentration for 25-Desacetyl Rifampicin

QC H (8.00 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	1750000	165000	10.6
Injection 2	1660000	162000	10.2
Injection 3	1720000	179000	9.61
Injection 4	1820000	172000	10.6
Injection 5	1820000	182000	10.0
Injection 6	1720000	167000	10.3
Average	1748333	171167	10.2
STDEV	62743	7985	0.376
% CV	3.6	4.7	3.7
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	1520000	163000	9.33
Injection 8	1690000	173000	9.77
Injection 9	1680000	168000	10.0
Injection 10	1770000	168000	10.5
Injection 11	1810000	161000	11.2
Injection 12	1870000	176000	10.6
Average	1723333	168167	10.2
STDEV	122909	5707	0.686
% CV	7.1	3.4	6.7
% Difference after Re-injection 1			**0.3**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	1490000	137000	10.9
Injection 14	1520000	137000	11.1
Injection 15	1400000	135000	10.4
Injection 16	1460000	139000	10.5
Injection 17	1370000	121000	11.3
Injection 18	1340000	127000	10.6
Average	1430000	132667	10.8
STDEV	70993	7090	0.374
% CV	5.0	5.3	3.5
% Difference after Re-injection 2			**5.5**

Table 6.52: Autosampler stability for extracted samples: Low concentration for 25-Desacetyl Rifampicin

QC L (0.0860 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	18500	127000	0.146
Injection 2	19900	128000	0.155
Injection 3	19900	128000	0.155
Injection 4	23400	130000	0.180
Injection 5	19600	135000	0.145
Injection 6	23200	131000	0.177
Average	20750	129833	0.160
STDEV	2042	2927	0.0152
% CV	9.8	2.3	9.5
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	21000	127000	0.165
Injection 8	19100	130000	0.147
Injection 9	21800	134000	0.163
Injection 10	20600	134000	0.154
Injection 11	22500	130000	0.173
Injection 12	24100	142000	0.170
Average	21517	132833	0.162
STDEV	1713	5231	0.00990
% CV	8.0	3.9	6.1
% Difference after Re-injection 1			**1.3**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	17600	121000	0.145
Injection 14	16100	112000	0.144
Injection 15	16800	111000	0.151
Injection 16	16300	101000	0.161
Injection 17	16100	109000	0.148
Injection 18	15200	95800	0.159
Average	16350	108300	0.151
STDEV	802	8858	0.00721
% CV	4.9	8.2	4.8
% Difference after Re-injection 2			**-5.3**

Table 6.53: Autosampler stability for extracted samples: High concentration for Ethambutol

QC H (8.00 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	1700000	124000	13.7
Injection 2	1640000	119000	13.8
Injection 3	1660000	125000	13.3
Injection 4	1780000	114000	15.6
Injection 5	1660000	118000	14.1
Injection 6	1660000	117000	14.2
Average	1683333	119500	14.1
STDEV	51251	4231	0.803
% CV	3.0	3.5	5.7
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	2800000	189000	14.8
Injection 8	2740000	186000	14.7
Injection 9	2670000	182000	14.7
Injection 10	2440000	171000	14.3
Injection 11	2450000	168000	14.6
Injection 12	2410000	176000	13.7
Average	2585000	178667	14.5
STDEV	171668	8383	0.420
% CV	6.6	4.7	2.9
		% Difference after Re-injection 1	**2.5**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	3320000	230000	14.4
Injection 14	3130000	235000	13.3
Injection 15	3230000	219000	14.7
Injection 16	3010000	203000	14.8
Injection 17	2770000	204000	13.6
Injection 18	2890000	208000	13.9
Average	3058333	216500	14.1
STDEV	208271	13722	0.629
% CV	6.8	6.3	4.5
		% Difference after Re-injection 2	**0.2**

Table 6.54: Autosampler stability for extracted samples: Low concentration for Ethambutol

QC L (0.0430 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	24300	170000	0.143
Injection 2	22900	151000	0.152
Injection 3	26000	154000	0.169
Injection 4	26600	154000	0.173
Injection 5	24200	155000	0.156
Injection 6	21700	151000	0.144
Average	24283	155833	0.156
STDEV	1839	7139	0.01253
% CV	7.6	4.6	8.0
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	38700	254000	0.152
Injection 8	35400	235000	0.151
Injection 9	38800	223000	0.174
Injection 10	36100	213000	0.169
Injection 11	35700	221000	0.162
Injection 12	33100	212000	0.156
Average	36300	226333	0.161
STDEV	2166	15895	0.00945
% CV	6.0	7.0	5.9
		% Difference after Re-injection 1	**3.0**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	45200	294000	0.154
Injection 14	46900	291000	0.161
Injection 15	43200	263000	0.164
Injection 16	43400	259000	0.168
Injection 17	42300	255000	0.166
Injection 18	41100	247000	0.166
Average	43683	268167	0.163
STDEV	2076	19600	0.00512
% CV	4.8	7.3	3.1
		% Difference after Re-injection 2	**4.6**

Table 6.55: Autosampler stability for extracted samples: High concentration for Isoniazid

QC H (20.0 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	1290000	165000	7.82
Injection 2	1330000	177000	7.51
Injection 3	1360000	174000	7.82
Injection 4	1420000	184000	7.72
Injection 5	1440000	189000	7.62
Injection 6	1420000	185000	7.68
Average	1376667	179000	7.69
STDEV	59554	8786	0.118
% CV	4.3	4.9	1.5
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	2240000	301000	7.44
Injection 8	2130000	292000	7.29
Injection 9	2080000	275000	7.56
Injection 10	2000000	276000	7.25
Injection 11	1990000	265000	7.51
Injection 12	1970000	269000	7.32
Average	2068333	279667	7.40
STDEV	103811	13938	0.127
% CV	5.0	5.0	1.7
		% Difference after Re-injection 1	**-3.9**
Validation 1 Re-injection	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	2180000	296000	7.36
Injection 14	2130000	296000	7.20
Injection 15	2080000	289000	7.20
Injection 16	2050000	289000	7.09
Injection 17	2000000	282000	7.09
Injection 18	2010000	285000	7.05
Average	2075000	289500	7.17
STDEV	70071	5683	0.114
% CV	3.4	2.0	1.6
		% Difference after Re-injection 2	**-6.9**

Table 6.56: Autosampler stability for extracted samples: Low concentration for Isoniazid

QC L (0.215 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	12700	171000	0.0743
Injection 2	13300	168000	0.0792
Injection 3	15200	182000	0.0835
Injection 4	15300	186000	0.0823
Injection 5	17000	187000	0.0909
Injection 6	16700	185000	0.0903
Average	15033	179833	0.0834
STDEV	1743	8232	0.00642
% CV	11.6	4.6	7.7
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	32200	301000	0.107
Injection 8	31300	294000	0.106
Injection 9	28900	270000	0.107
Injection 10	28800	267000	0.108
Injection 11	27600	268000	0.103
Injection 12	27900	248000	0.113
Average	29450	274667	0.107
STDEV	1873	19511	0.00306
% CV	6.4	7.1	2.9
		% Difference after Re-injection 1	**28.7**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	32800	301000	0.109
Injection 14	32200	293000	0.110
Injection 15	35700	287000	0.124
Injection 16	28200	290000	0.0972
Injection 17	32600	280000	0.116
Injection 18	32000	267000	0.120
Average	32250	286333	0.113
STDEV	2400	11725	0.00962
% CV	7.4	4.1	8.5
		% Difference after Re-injection 2	**35.3**

Table 6.57: Autosampler stability for extracted samples: High concentration for Pyrazinamide

QC H (64.0 µg/ml)			
Validation 1 Batch End	Peak area	ISTD peak area	Ratio
Injection 1	3240000	279000	11.6
Injection 2	3670000	330000	11.1
Injection 3	4160000	357000	11.7
Injection 4	4290000	377000	11.4
Injection 5	4530000	414000	10.9
Injection 6	4980000	414000	12.0
Average	4145000	361833	11.5
STDEV	618280	52098	0.393
% CV	14.9	14.4	3.4
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	7930000	729000	10.9
Injection 8	7520000	703000	10.7
Injection 9	7560000	701000	10.8
Injection 10	7510000	692000	10.9
Injection 11	7430000	654000	11.4
Injection 12	7420000	672000	11.0
Average	7561667	691833	10.9
STDEV	188406	26134	0.238
% CV	2.5	3.8	2.2
		% Difference after Re-injection 1	**-4.5**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	8030000	798000	10.1
Injection 14	8370000	816000	10.3
Injection 15	8050000	769000	10.5
Injection 16	7860000	760000	10.3
Injection 17	7970000	745000	10.7
Injection 18	7900000	741000	10.7
Average	8030000	771500	10.4
STDEV	181879	29845	0.244
% CV	2.3	3.9	2.3
		% Difference after Re-injection 2	**-9.1**

Table 6.58: Autosampler stability for extracted samples: Low concentration for Pyrazinamide

QC L (0.688 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	42300	305000	0.139
Injection 2	50600	354000	0.143
Injection 3	53200	381000	0.140
Injection 4	57100	430000	0.133
Injection 5	59900	434000	0.138
Injection 6	64900	446000	0.146
Average	54667	391667	0.140
STDEV	7870	55240	0.00438
% CV	14.4	14.1	3.1
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	113000	792000	0.143
Injection 8	104000	780000	0.133
Injection 9	108000	756000	0.143
Injection 10	100000	728000	0.137
Injection 11	109000	741000	0.147
Injection 12	104000	701000	0.148
Average	106333	749667	0.142
STDEV	4590	33661	0.00573
% CV	4.3	4.5	4.0
% Difference after Re-injection 1			**1.7**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	121000	867000	0.140
Injection 14	117000	875000	0.134
Injection 15	111000	804000	0.138
Injection 16	115000	807000	0.143
Injection 17	114000	827000	0.138
Injection 18	121000	792000	0.153
Average	116500	828667	0.141
STDEV	3987	34760	0.00655
% CV	3.4	4.2	4.7
% Difference after Re-injection 2			**0.8**

Table 6.59: Autosampler stability for extracted samples: High concentration for Rifampicin

QC H (24.0 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	7660000	2100000	3.65
Injection 2	7390000	2060000	3.59
Injection 3	7650000	2190000	3.49
Injection 4	7590000	2150000	3.53
Injection 5	7450000	2120000	3.51
Injection 6	7430000	2120000	3.50
Average	7528333	2123333	3.55
STDEV	119066	44121	0.060
% CV	1.6	2.1	1.7
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	5980000	1650000	3.62
Injection 8	6020000	1680000	3.58
Injection 9	5860000	1640000	3.57
Injection 10	5970000	1690000	3.53
Injection 11	5940000	1610000	3.69
Injection 12	5940000	1630000	3.64
Average	5951667	1650000	3.61
STDEV	53821	30332	0.056
% CV	0.9	1.8	1.6
		% Difference after Re-injection 1	**1.7**
Validation Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	4960000	1340000	3.70
Injection 14	4990000	1340000	3.72
Injection 15	5060000	1390000	3.64
Injection 16	4960000	1360000	3.65
Injection 17	4890000	1340000	3.65
Injection 18	4980000	1350000	3.69
Average	4973333	1353333	3.68
STDEV	55015	19664	0.034
% CV	1.1	1.5	0.9
		% Difference after Re-injection 2	**3.6**

Table 6.60: Autosampler stability for extracted samples: Low concentration for Rifampicin

QC L (0.258 µg/ml)			
Validation 1 Batch End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	101000	2290000	0.0441
Injection 2	92000	2130000	0.0432
Injection 3	99000	2250000	0.0440
Injection 4	99600	2240000	0.0445
Injection 5	98500	2160000	0.0456
Injection 6	101000	2110000	0.0479
Average	98517	2196667	0.0449
STDEV	3353	73121	0.00166
% CV	3.4	3.3	3.7
Validation 1 Re-injection 1	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	72100	1600000	0.0451
Injection 8	60400	1410000	0.0428
Injection 9	75400	1610000	0.0468
Injection 10	73700	1620000	0.0455
Injection 11	74700	1680000	0.0445
Injection 12	82000	1660000	0.0494
Average	73050	1596667	0.0457
STDEV	7067	96471	0.00224
% CV	9.7	6.0	4.9
% Difference after Re-injection 1			**1.8**
Validation 1 Re-injection 2	**Peak area**	**ISTD peak area**	**Ratio**
Injection 13	59100	1360000	0.0435
Injection 14	52300	1120000	0.0467
Injection 15	56700	1240000	0.0457
Injection 16	53000	1150000	0.0461
Injection 17	54400	1240000	0.0439
Injection 18	55500	1190000	0.0466
Average	55167	1216667	0.0454
STDEV	2507	85010	0.00141
% CV	4.5	7.0	3.1
% Difference after Re-injection 2			**1.2**

The CV(%) and % Difference for all analytes except INH were within 15% after both re-injections, indicating that the internal standards compensate for any changes in the assay for these analytes. Absolute on-instrument stability for these analytes was indicated for at least 50

hours. For INH, the % Difference after reinjection was 28.7 % at ~26 hours and 35.3 % after ~ 50 hours, indicating instability.

6.4.8. Whole blood stability

Previous reports indicate that AcINH INH are stable in whole blood for one and two hours respectively when kept on ice (VL2010-125 V3.2). PZA and RIF are stable in whole blood for two hours at room temperature (VL2012-144 V2.1, VL2004-107 V7.1). The stability of desRIF and EMB in whole blood will be determined at a later stage.

6.5. Recovery

The extraction recovery pertains to the extraction efficiency of the analytical process within the limits of variability. It was determined by comparing the analytical response of blank matrix spiked with the analyte then extracted with the response of the blank matrix first extracted and then spiked with analyte (theoretical, represents 100% recovery).

Acceptance criteria: The recovery of a quantitative drug assay method should be consistent, and the precision of the measured recovery, expressed as percentage coefficient of variation, should not exceed 15% for any concentration of the analyte at which it is determined. Recovery reproducibility between concentration levels should not be >15%.

a. Extracted (test) samples: Six QCs at each concentration level in six different lots of matrix (low, medium and high) were extracted as per the analytical method.

b. Theoretical samples: Samples from the same sources as above were spiked with analytes post extraction, at each QC concentration level (relative to the final concentration of the corresponding extracted QC's level) in six-fold.

The analyte peak areas found after extraction were compared to the theoretical peak area and were expressed as a percentage recovery. The results of the recovery experiments are shown in Tables 6.61- 6.66 below.

Table 6.61: AcINH recovery

	High Concentration (10.0 µg/ml)		**Medium Concentration (5.00 µg/ml)**		**Low Concentration (0.107 µg/ml)**	
	Extracted: Peak Area Ratio	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**
Sample 1	6.26	5.32	3.03	2.74	0.318	0.271
Sample 2	5.99	5.5	2.95	2.74	0.318	0.322
Sample 3	5.98	5.89	3	2.74	0.315	0.317
Sample 4	5.91	5.48	2.87	2.75	0.311	0.297
Sample 5	6.06	5.47	2.99	2.86	0.288	0.313
Sample 6	6.1	5.47	3.1	2.85	0.319	0.303
Average	6.05	5.52	2.99	2.78	0.312	0.304
STDEV	0.122	0.192	0.0772	0.0583	0.0119	0.0185
% CV	2	3.5	2.6	2.1	3.8	6.1
% Recovery		109.6		107.6		102.5
				Average % Recovery		106.5
				Average % CV		3.4

Table 6.62: desRIF recovery

	High Concentration (8.00 µg/ml)		**Medium Concentration (4.00 µg/ml)**		**Low Concentration (0.0860 µg/ml)**	
	Extracted: Peak Area Ratio	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**
Sample 1	10.5	8.97	6.31	5.15	0.873	0.605
Sample 2	10.6	9.19	5.85	4.93	0.823	0.645
Sample 3	10.6	9.19	5.98	4.89	0.909	0.659
Sample 4	10.7	8.83	6.01	4.96	0.795	0.704
Sample 5	10.7	8.59	6.31	5.45	0.84	0.656
Sample 6	10.7	8.34	6.21	4.61	0.806	0.702
Average	10.6	8.85	6.11	5	0.841	0.662
STDEV	0.0816	0.339	0.192	0.281	0.0432	0.0373
% CV	0.8	3.8	3.1	5.6	5.1	5.6
% Recovery		120.1		122.3		127.1
				Average % Recovery		123.2
				Average % CV		2.9

Table 6.63: EMB recovery

	High Concentration (4.00 µg/ml)		**Medium Concentration (2.00 µg/ml)**		**Low Concentration (0.0430 µg/ml)**	
	Extracted: Peak Area Ratio	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**
Sample 1	14.4	13.4	8.08	7.26	0.834	0.822
Sample 2	15.9	14.6	8.59	7.32	0.845	0.862
Sample 3	14.4	14.7	8.32	7.58	0.859	0.89
Sample 4	15.3	15	8.7	7.18	0.862	0.888
Sample 5	15.5	14.2	8.26	7.88	0.886	0.815
Sample 6	14.5	15.1	7.86	7.37	0.879	0.857
Average	15	14.5	8.3	7.43	0.861	0.856
STDEV	0.651	0.626	0.312	0.258	0.0197	0.0318
% CV	4.3	4.3	3.8	3.5	2.3	3.7
% Recovery		103.4		111.7		100.6
				Average % Recovery		105.3
				Average % CV		5.5

Table 6.64: INH recovery

	High Concentration (20.0 µg/ml)		**Medium Concentration (10.0 µg/ml)**		**Low Concentration (0.215 µg/ml)**	
	Extracted: Peak Area Ratio	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**	**Extracted: Peak Area Ratio**	**Theoretical: Peak Area Ratio**
Sample 1	5.66	4.71	2.54	2.36	0.306	0.281
Sample 2	5.43	4.68	2.8	2.52	0.285	0.311
Sample 3	5.4	4.92	2.6	2.44	0.346	0.295
Sample 4	5.58	5	2.65	2.3	0.303	0.29
Sample 5	5.14	4.85	2.65	2.3	0.322	0.299
Sample 6	5.35	4.95	2.66	2.23	0.311	0.291
Average	5.43	4.85	2.65	2.36	0.312	0.295
STDEV	0.183	0.131	0.0863	0.106	0.0205	0.0101
% CV	3.4	2.7	3.3	4.5	6.6	3.4
% Recovery		111.9		112.4		106
				Average % Recovery		110.1
				Average % CV		3.2

Table 6.65: Pyrazinamide recovery

	High Concentration (64.0 µg/ml)		**Medium Concentration (32.0 µg/ml)**		**Low Concentration (0.688 µg/ml)**	
	Extraction: Peak Area Ratio	**Spiked Blank: Peak Area Ratio**	**Extraction: Peak Area Ratio**	**Spiked Blank: Peak Area Ratio**	**Extraction: Peak Area Ratio**	**Spiked Blank: Peak Area Ratio**
Sample 1	13	9.6	6.93	5.15	0.841	0.609
Sample 2	13.3	9.7	7.27	5.11	0.822	0.691
Sample 3	12.7	10.1	6.82	5.31	0.853	0.651
Sample 4	12.7	10.1	6.98	5.33	0.829	0.655
Sample 5	12.9	9.7	7.05	5.27	0.851	0.676
Sample 6	13.2	9.6	7.21	5.14	0.856	0.649
Average	13	9.8	7.04	5.22	0.842	0.655
STDEV	0.25	0.251	0.171	0.096	0.0139	0.0279
% CV	1.9	2.6	2.4	1.8	1.7	4.3
% Recovery		132.6		135		128.5
				Average % Recovery	132	
				Average % CV	2.5	

Table 6.66: RIF recovery

	High Concentration (24.0 µg/ml)		**Medium Concentration (12.0 µg/ml)**		**Low Concentration (0.258 µg/ml)**	
	Extraction: Peak Area Ratio	**Spiked Blank: Peak Area Ratio**	**Extraction: Peak Area Ratio**	**Spiked Blank: Peak Area Ratio**	**Extraction: Peak Area Ratio**	**Spiked Blank: Peak Area Ratio**
Sample 1	2.9	2.03	1.49	1.18	0.175	0.128
Sample 2	2.2	2.08	1.49	1.2	0.17	0.15
Sample 3	2.88	2.14	1.48	1.15	0.178	0.145
Sample 4	2.86	2.11	1.48	1.16	0.179	0.143
Sample 5	2.9	2.07	1.48	1.16	0.172	0.145
Sample 6	2.79	2.11	1.56	1.12	0.168	0.14
Average	2.76	2.09	1.5	1.16	0.174	0.142
STDEV	0.275	0.038	0.0314	0.027	0.0044	0.0075
% CV	10	1.8	2.1	2.3	2.5	5.3
% Recovery		131.8		128.8		122.4
				Average % Recovery		127.7
				Average % CV		3.8

The mean recovery of AcINH, desRIF, EMB, INH, PZA and RIF from six different lots of plasma over the calibration range was 106.5, 123.2, 105.3, 110.1, 132.0 and 127.7% respectively with CV (%) of 3.4, 2.9, 5.5, 3.2, 2.5 and 3.8% respectively which was within acceptable limits. All analyte recoveries were above 100%. For three of the analytes (AcINH, EMB and INH) the recovery was less than 115%, which could be a result of random analytical errors when the actual recovery was approximately 100%. For analytes with recoveries greater than 120% (desRIF, PZA and RIF), ion enhancement could be a possible reason, although nit

is not clear why matrix effects (ion enhancement) would be different when the same matrix was used.

6.6. Matrix effects

In biological analyses, matrix refers to endogenous and non-endogenous components of a sample other than the analyte being studied. The presence of certain matrix components may affect analyte and/or internal standard ionization. Factors such as haemolysis and anticoagulant type may also affect ionization. An appropriate ISTD which adequately resembles analyte behaviour compensates, to some extent, for matrix effects.

Acceptance criteria: The analyte peak areas for each concentration level in an individual plasma sample are used to generate regressions for that sample. The slope variability (%CV) for the six different matrix sources should not be >5%.

Plasma from 6 different sources were extracted (without ISTD). Each plasma sample was spiked at low, medium and high concentration levels (accounting for any calculations for dilutions in the analytical method). The results of the regression slopes were calculated in Tables 6.67 – 6.72.

Table 6.67: AcINH regression results from 6 different matrices

	High Conc. 10.0 µg/ml Peak Area Ratio	**Medium Conc. 5.00 µg/ml Peak Area Ratio**	**Low Conc. 0.107 µg/ml Peak Area Ratio**	**Area Ratio v Conc. Regression Slope**
15-11-2017-32E	5.320	2.730	0.270	0.511
15-11-2017-25E	5.500	2.730	0.323	0.523
15-11-2017-33E	5.900	2.730	0.317	0.564
15-11-2017-15E	5.480	2.750	0.296	0.524
15-11-2017-29E	5.460	2.860	0.314	0.521
15-11-2017-30E	5.470	2.860	0.303	0.522
Average	5.520	2.780	0.304	0.527
STDEV	0.195	0.0658	0.0192	0.0187
% CV	3.5	2.4	6.3	3.5

Table 6.68: desRIF regression results from 6 different matrices

	High Conc. 8.00 µg/ml Peak Area Ratio	**Medium Conc. 4.00 µg/ml Peak Area Ratio**	**Low Conc. 0.0860 µg/ml Peak Area Ratio**	**Area Ratio v Conc. Regression Slope**
15-11-2017-32E	8.99	5.16	0.603	1.06
15-11-2017-25E	9.17	4.92	0.645	1.08
15-11-2017-33E	9.21	4.9	0.662	1.08
15-11-2017-15E	8.81	4.97	0.704	1.02
15-11-2017-29E	8.6	5.46	0.656	1.00
15-11-2017-30E	8.36	4.6	0.704	0.968
Average	8.86	5	0.662	1.04
STDEV	0.333	0.287	0.0383	0.045
% CV	3.8	5.7	5.8	4.3

Table 6.69: EMB regression results from 6 different matrices

	High Conc. 4.00 µg/ml Peak Area Ratio	**Medium Conc. 2.00 µg/ml Peak Area Ratio**	**Low Conc. 0.0430 µg/ml Peak Area Ratio**	**Area Ratio v Conc. Regression Slope**
15-11-2017-32E	13.4	7.23	0.824	3.19
15-11-2017-25E	14.7	7.34	0.859	3.5
15-11-2017-33E	14.7	7.61	0.892	3.49
15-11-2017-15E	15.1	7.19	0.885	3.6
15-11-2017-29E	14.2	7.85	0.815	3.38
15-11-2017-30E	15.1	7.35	0.86	3.6
Average	14.5	7.43	0.856	3.46
STDEV	0.639	0.253	0.0313	0.156
% CV	4.4	3.4	3.7	4.5

Table 6.70: INH regression results from 6 different matrices

	High Conc. 20.0 µg/ml Peak Area Ratio	**Medium Conc. 10.0 µg/ml Peak Area Ratio**	**Low Conc. 0.215 µg/ml Peak Area Ratio**	**Area Ratio v Conc. Regression Slope**
15-11-2017-32E	4.69	2.36	0.281	0.223
15-11-2017-25E	4.69	2.53	0.31	0.221
15-11-2017-33E	4.91	2.44	0.295	0.233
15-11-2017-15E	5.02	2.3	0.289	0.239
15-11-2017-29E	4.870	2.300	0.300	0.231
15-11-2017-30E	4.930	2.240	0.290	0.235
Average	4.850	2.360	0.294	0.230
STDEV	0.136	0.106	0.0102	0.00703
% CV	2.8	4.5	3.5	3.0

Table 6.71: PZA regression results from 6 different matrices

	High Conc. 64.0 µg/ml Peak Area Ratio	**Medium Conc. 32.0 µg/ml Peak Area Ratio**	**Low Conc. 0.688 µg/ml Peak Area Ratio**	**Area Ratio v Conc. Regression Slope**
15-11-2017-32E	9.6	5.15	0.608	0.142
15-11-2017-25E	9.7	5.11	0.691	0.142
15-11-2017-33E	10.1	5.32	0.651	0.15
15-11-2017-15E	10.1	5.32	0.656	0.149
15-11-2017-29E	9.7	5.26	0.676	0.142
15-11-2017-30E	9.6	5.14	0.649	0.141
Average	9.8	5.22	0.655	0.144
STDEV	0.244	0.0966	0.0285	0.00382
% CV	2.5	1.9	4.3	2.7

Table 6.72: RIF regression results from 6 different matrices

	High Conc. 24.0 µg/ml Peak Area Ratio	**Medium Conc. 12.0 µg/ml Peak Area Ratio**	**Low Conc. 0.258 µg/ml Peak Area Ratio**	**Area Ratio v Conc. Regression Slope**
15-11-2017-32E	2.03	1.18	0.128	0.0801
15-11-2017-25E	2.08	1.2	0.151	0.0811
15-11-2017-33E	2.14	1.15	0.145	0.0839
15-11-2017-15E	2.11	1.16	0.144	0.0828
15-11-2017-29E	2.07	1.16	0.145	0.0811
15-11-2017-30E	2.12	1.12	0.14	0.0833
Average	2.09	1.16	0.142	0.0821
STDEV	0.039	0.027	0.00758	0.00151
% CV	1.9	2.3	5.3	1.8

The slope variability (%CV) for six different K3EDTA plasma samples was 3.5, 4.3, 4.5, 3.0, 2.7 and 1.8% for AcINH, desRIF, EMB, INH, PZA and RIF, respectively. These results indicate that matrix effects do not adversely influence the precision of the assay.

6.7. Effect of haemolysis

The presence of haemolysed blood in samples could affect the ionization of the analyte and ISTD during assay. If ionisation suppression of the ISTD is comparable to that of the analyte, the ISTD is regarded as providing sufficient compensation for the analyte. Haemolysis was tested using 2% haemolysed blood in plasma. The ability of the internal standard to compensate for haemolysis effect on analyte determination was assessed by comparing normal and haemolysed plasma response ratios at high and low concentrations (Tables 6.73 – 6.78).

Table 6.73: Effect of 2% Haemolysis: AcINH

	High Concentration (10.0 µg/ml) Peak Area Ratio		**Med Concentration (5.00 µg/ml) Peak Area Ratio**		**Low Concentration (0.107 µg/ml) Peak Area Ratio**	
	Normal	**2% Haemolysed**	**Normal**	**2% Haemolysed**	**Normal**	**2% Haemolysed**
Sample 1	7.05	6.46	3.4	3.35	0.0715	0.0623
Sample 2	6.72	6.38	3.48	3.25	0.07	0.0628
Sample 3		6.33		3.08		0.0654
Sample 4		6.43		3.28		0.0614
Sample 5		6.31		3.18		0.059
Sample 6		6.24		3.21		0.0615
Average	6.89	6.36	3.44	3.23	0.0708	0.0621
STDEV	0.233	0.081	0.0566	0.0922	0.00106	0.00209
% CV	3.4	1.3	1.6	2.9	1.5	3.4

% Difference		-7.6		-6.3		-12.3

Table 6.74: Effect of 2% Haemolysis: desRIF

	High Concentration (8.00 µg/ml) Peak Area Ratio		**Med Concentration (4.00 µg/ml) Peak Area Ratio**		**Low Concentration (0.0860 µg/ml) Peak Area Ratio**	
	Normal	**2% Haemolysed**	**Normal**	**2% Haemolysed**	**Normal**	**2% Haemolysed**
Sample 1	12.7	12.6	7.59	6.81	0.189	0.162
Sample 2	13.1	12.4	7.5	7.05	0.2	0.155
Sample 3		11.5		6.7		0.169
Sample 4		11.5		6.68		0.159
Sample 5		11.5		6.84		0.182
Sample 6		11		7.25		0.155
Average	12.9	11.8	7.55	6.89	0.195	0.164
STDEV	0.283	0.616	0.0636	0.221	0.00778	0.0104
% CV	2.2	5.2	0.8	3.2	4	6.3
% Difference		-8.9		-8.7		-15.9

Table 6.75: Effect of 2% Haemolysis: EMB

	High Concentration (4.00 µg/ml) Peak Area Ratio		**Med Concentration (2.00 µg/ml) Peak Area Ratio**		**Low Concentration (0.0430 µg/ml) Peak Area Ratio**	
	Normal	**2% Haemolysed**	**Normal**	**2% Haemolysed**	**Normal**	**2% Haemolysed**
Sample 1	16.4	14.1	8.07	7.82	0.184	0.152
Sample 2	16.6	14.7	8.44	7.53	0.199	0.168

Sample 3		14.5		7.82		0.15
Sample 4		15.1		7.67		0.156
Sample 5		15		7.92		0.154
Sample 6		14		7.5		0.153
Average	16.5	14.6	8.26	7.71	0.192	0.156
STDEV	0.141	0.455	0.262	0.171	0.0106	0.0064
% CV	0.9	3.1	3.2	2.2	5.5	4.1
% Difference		-11.7		-6.6		-18.8

Table 6.76: Effect of 2% Haemolysis: INH

	High Concentration (20.0 µg/ml) Peak Area Ratio		**Med Concentration (10.0 µg/ml) Peak Area Ratio**		**Low Concentration (0.215 µg/ml) Peak Area Ratio**	
	Normal	**2% Haemolysed**	**Normal**	**2% Haemolysed**	**Normal**	**2% Haemolysed**
Sample 1	7.81	7.57	3.74	3.61	0.158	0.157
Sample 2	7.87	7.28	3.73	3.61	0.145	0.171
Sample 3		7.21		3.56		0.161
Sample 4		7.39		3.64		0.167
Sample 5		6.87		3.76		0.168
Sample 6		7.36		3.59		0.154
Average	7.84	7.28	3.74	3.63	0.152	0.163
STDEV	0.042	0.235	0.00707	0.0697	0.00919	0.00672
% CV	0.5	3.2	0.2	1.9	6.1	4.1
% Difference		-7.1		-2.9		7.6

Table 6.77: Effect of 2% Haemolysis: PZA

	High Concentration (64.0 µg/ml) Peak Area Ratio		**Med Concentration (32.0 µg/ml) Peak Area Ratio**		**Low Concentration (0.688 µg/ml) Peak Area Ratio**	
	Normal	**2% Haemolysed**	**Normal**	**2% Haemolysed**	**Normal**	**2% Haemolysed**
Sample 1	11.5	10.5	6.13	5.87	0.161	0.121

Sample 2	11.7	10.2	6.48	5.69	0.151	0.132
Sample 3		10.5		5.44		0.131
Sample 4		10.5		6.02		0.124
Sample 5		10.5		5.96		0.131
Sample 6		10.6		5.84		0.121
Average	11.6	10.5	6.31	5.8	0.156	0.127
STDEV	0.141	0.137	0.247	0.2108	0.00707	0.00524
% CV	1.2	1.3	3.9	3.6	4.5	4.1
% Difference		-9.8		-8		-18.8

Table 6.78: Effect of 2% Haemolysis: RIF

	High Concentration (24.0 µg/ml) Peak Area Ratio		**Med Concentration (12.0 µg/ml) Peak Area Ratio**		**Low Concentration (0.258 µg/ml) Peak Area Ratio**	
	Normal	**2% Haemolysed**	**Normal**	**2% Haemolysed**	**Normal**	**2% Haemolysed**
Sample 1	3.6	3.15	1.81	1.72	0.0399	0.034
Sample 2	3.33	3.23	1.82	1.7	0.041	0.0348
Sample 3		3.24		1.67		0.0353
Sample 4		3.25		1.76		0.034
Sample 5		3.1		1.78		0.0358
Sample 6		3.22		1.76		0.0345
Average	3.47	3.2	1.82	1.73	0.0405	0.0347
STDEV	0.191	0.0598	0.00707	0.0422	0.000778	0.00072
% CV	5.5	1.9	0.4	2.4	1.9	2.1
% Difference		-7.7		-4.6		-14.1

Acceptance criteria: A % Difference higher than 15% between the peak area ratios observed in haemolysed samples and normal samples and a high CV(%) (higher than 15%) indicates that haemolysis has an effect on the assay of the analyte and that the internal standard does not sufficiently compensate for the analyte.

The % Differences were within 15% for three of the six analytes (AcINH, INH and RIF) in 2% haemolysed plasma samples, therefore haemolysis has no significant effect on the quantification of these analytes. The % Difference for desRIF, EMB and PZA was greater than 15% at low concentrations indicating that low concentrations of these analytes cannot be reliably measured in plasma samples that are 2% haemolysed.

6.8. Matrix anticoagulant effect

To evaluate the influence of the matrix anticoagulant on the analyte and internal standard ionization and accuracy, a set of quality controls prepared using plasma containing K2EDTA as the anticoagulant, were compared to those in which K3EDTA was the anticoagulant, in a validation batch. The results are presented in Tables 6.79 – 6.84.

Table 6.79: Effect of K2EDTA anticoagulant on AcINH

	High Concentration (10.0 µg/ml) Peak Area Ratio		**Med Concentration (5.00 µg/ml) Peak Area Ratio**		**Low Concentration (0.107 µg/ml) Peak Area Ratio**	
	K3EDTA	**K2EDTA**	**K3EDTA**	**K2EDTA**	**K3EDTA**	**K2EDTA**
Sample 1	7.05	6.15	3.4	3.28	0.0715	0.0702
Sample 2	6.72	6.14	3.48	3.35	0.07	0.0628
Sample 3		6.17		3.32		0.0664
Sample 4		6.44		3.28		0.0638
Sample 5		6.23		3.19		0.0713
Sample 6		6.25		3.18		0.068
Average	6.89	6.23	3.44	3.27	0.0708	0.0671
STDEV	0.233	0.112	0.0566	0.0686	0.00106	0.0034

% CV	3.4	1.8	1.6	2.1	1.5	5.1
% Difference		-9.5		-5		-5.2

Table 6.80: Effect of K2EDTA anticoagulant on desRIF

	High Concentration (8.00 µg/ml) Peak Area Ratio		**Med Concentration (4.00 µg/ml) Peak Area Ratio**		**Low Concentration (0.0860 µg/ml) Peak Area Ratio**	
	K3EDTA	**K2EDTA**	**K3EDTA**	**K2EDTA**	**K3EDTA**	**K2EDTA**
Sample 1	12.7	12.4	7.59	7.53	0.189	0.185
Sample 2	13.1	10.8	7.5	6.74	0.2	0.191
Sample 3		11.6		7.07		0.186
Sample 4		11.3		7.16		0.169
Sample 5		12.3		6.02		0.19
Sample 6		11		6.94		0.184
Average	12.9	11.6	7.55	6.91	0.195	0.184
STDEV	0.283	0.665	0.0636	0.509	0.00778	0.00794
% CV	2.2	5.8	0.8	7.4	4	4.3
% Difference		-10.3		-8.4		-5.3

Table 6.81: Effect of K2EDTA anticoagulant on EMB

	High Concentration (4.00 µg/ml) Peak Area Ratio	**Med Concentration (2.00 µg/ml) Peak Area Ratio**	**Low Concentration (0.0430 µg/ml) Peak Area Ratio**

	K3EDTA	K2EDTA	K3EDTA	K2EDTA	K3EDTA	K2EDTA
Sample 1	16.4	14.4	8.07	7.12	0.184	0.187
Sample 2	16.6	15	8.44	8.01	0.199	0.156
Sample 3		13.8		7.07		0.154
Sample 4		14.7		7.57		0.152
Sample 5		14.4		7.65		0.179
Sample 6		13.6		7.44		0.153
Average	16.5	14.3	8.26	7.48	0.192	0.164
STDEV	0.141	0.531	0.262	0.351	0.0106	0.0154
% CV	0.9	3.7	3.2	4.7	5.5	9.4
% Difference		-13.2		-9.4		-14.6

Table 6.82: Effect of K2EDTA anticoagulant on INH

	High Concentration (20.0 µg/ml) Peak Area Ratio		**Med Concentration (10.0 µg/ml) Peak Area Ratio**		**Low Concentration (0.215 µg/ml) Peak Area Ratio**	
	K3EDTA	**K2EDTA**	**K3EDTA**	**K2EDTA**	**K3EDTA**	**K2EDTA**
Sample 1	7.81	7.07	3.74	3.72	0.158	0.134
Sample 2	7.87	6.94	3.73	3.67	0.145	0.13
Sample 3		6.91		3.52		0.131
Sample 4		7		3.58		0.129
Sample 5		6.83		3.72		0.139
Sample 6		6.72		3.6		0.128
Average	7.84	6.91	3.74	3.64	0.152	0.132
STDEV	0.042	0.124	0.00707	0.0814	0.00919	0.00407
% CV	0.5	1.8	0.2	2.2	6.1	3.1
% Difference		-11.8		-2.7		-13

Table 6.83: Effect of K2EDTA anticoagulant on PZA

	High Concentration (64.0 µg/ml) Peak Area Ratio	**Med Concentration (32.0 µg/ml) Peak Area Ratio**	**Low Concentration (0.688 µg/ml) Peak Area Ratio**

	K3EDTA	K2EDTA	K3EDTA	K2EDTA	K3EDTA	K2EDTA
Sample 1	11.5	10.2	6.13	5.99	0.161	0.14
Sample 2	11.7	10.1	6.48	6.04	0.151	0.137
Sample 3		10		5.94		0.135
Sample 4		10.3		6.04		0.139
Sample 5		10.1		5.91		0.142
Sample 6		10.6		6.08		0.144
Average	11.6	10.2	6.31	6	0.156	0.14
STDEV	0.141	0.214	0.247	0.0654	0.00707	0.00327
% CV	1.2	2.1	3.9	1.1	4.5	2.3
% Difference		-11.9		-4.8		-10.6

Table 6.84: Effect of K2EDTA anticoagulant on RIF

	High Concentration (24.0 µg/ml) Peak Area Ratio		**Med Concentration (12.0 µg/ml) Peak Area Ratio**		**Low Concentration (0.258 µg/ml) Peak Area Ratio**	
	K3EDTA	**K2EDTA**	**K3EDTA**	**K2EDTA**	**K3EDTA**	**K2EDTA**
Sample 1	3.6	3.09	1.81	1.72	0.0399	0.039
Sample 2	3.33	3.06	1.82	1.74	0.041	0.0386
Sample 3		3.05		1.71		0.0395
Sample 4		3.1		1.69		0.0371
Sample 5		3.11		1.68		0.0354
Sample 6		3		1.71		0.036
Average	3.47	3.07	1.82	1.71	0.0405	0.0376
STDEV	0.191	0.0407	0.00707	0.0214	0.000778	0.00169
% CV	5.5	1.3	0.4	1.3	1.9	4.5
% Difference		-11.4		-5.9		-7

Acceptance criteria: A % Difference higher than 15% between the peak area ratios observed in quality controls prepared in plasma containing K2EDTA as the anticoagulant compared to

K3EDTA indicates that the type of anticoagulant used affects the assay and that the internal standard does not sufficiently compensate for the analyte.

The % Difference was less than 15% for the six analytes at high, medium and low concentrations, indicating that the use of K2EDTA vs K3EDTA anticoagulant has no significant influence on the precision and accuracy of the assay.

6.9. Linearity

Concentration was plotted against peak area ratio for each calibration standard to generate calibration curves for each analyte. Quadratic regressions were used for each analyte and were weighted by 1/concentration for all analytes except PZA, which was weighted by 1/concentration^2. Figure 6.73 to Figure 6.78 show representative calibration curves for each analyte.

AcINH

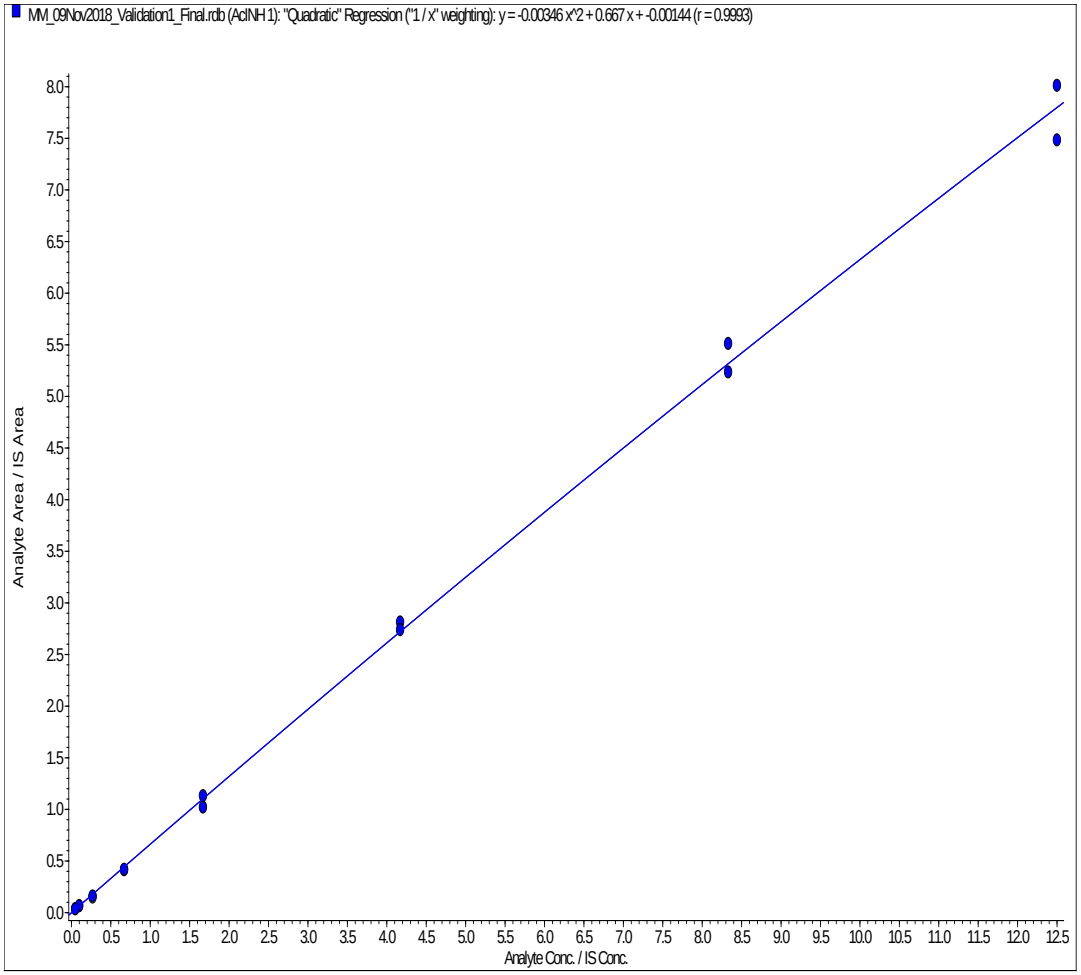

Figure 6.14: Representative calibration curve for AcINH: Validation 1, Day 1.

The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 6.85.

Table 6.85: Regression equation: AcINH

Validation Batch	**Quadratic Calibration Curve Parameters**			
	a	**b**	**c**	**r**
1	-0.00346	0.667	-0.00144	0.9993

desRIF

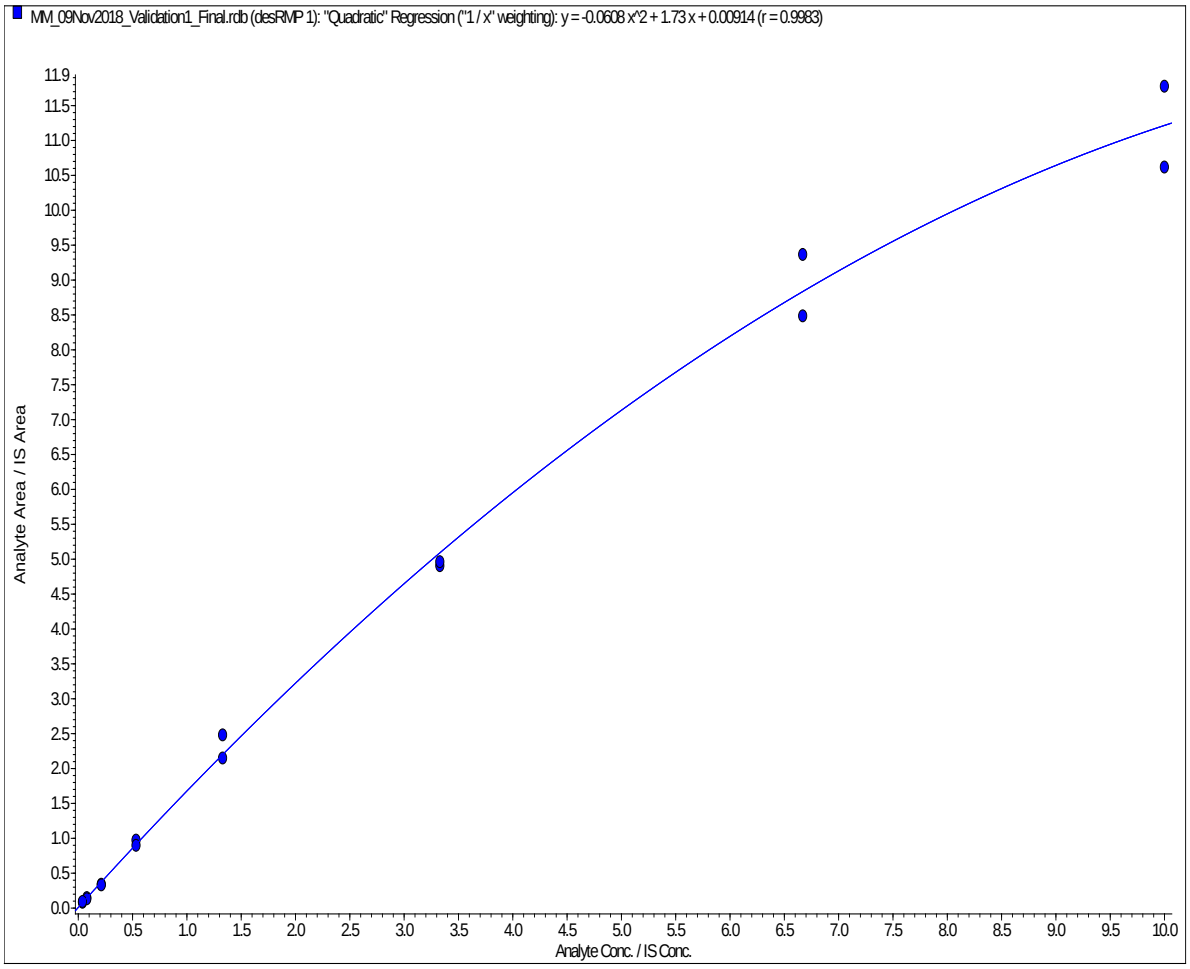

Figure 6.15: Representative calibration curve for 25-Desacetyl-Rifampicin: Validation 1, Day 1

The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 6.86.

Table 6.86: Regression equation: desRIF

Validation Batch	**Quadratic Calibration Curve Parameters**			
	a	**b**	**c**	**r**
1	-0.0608	1.73	0.00914	0.9983

EMB

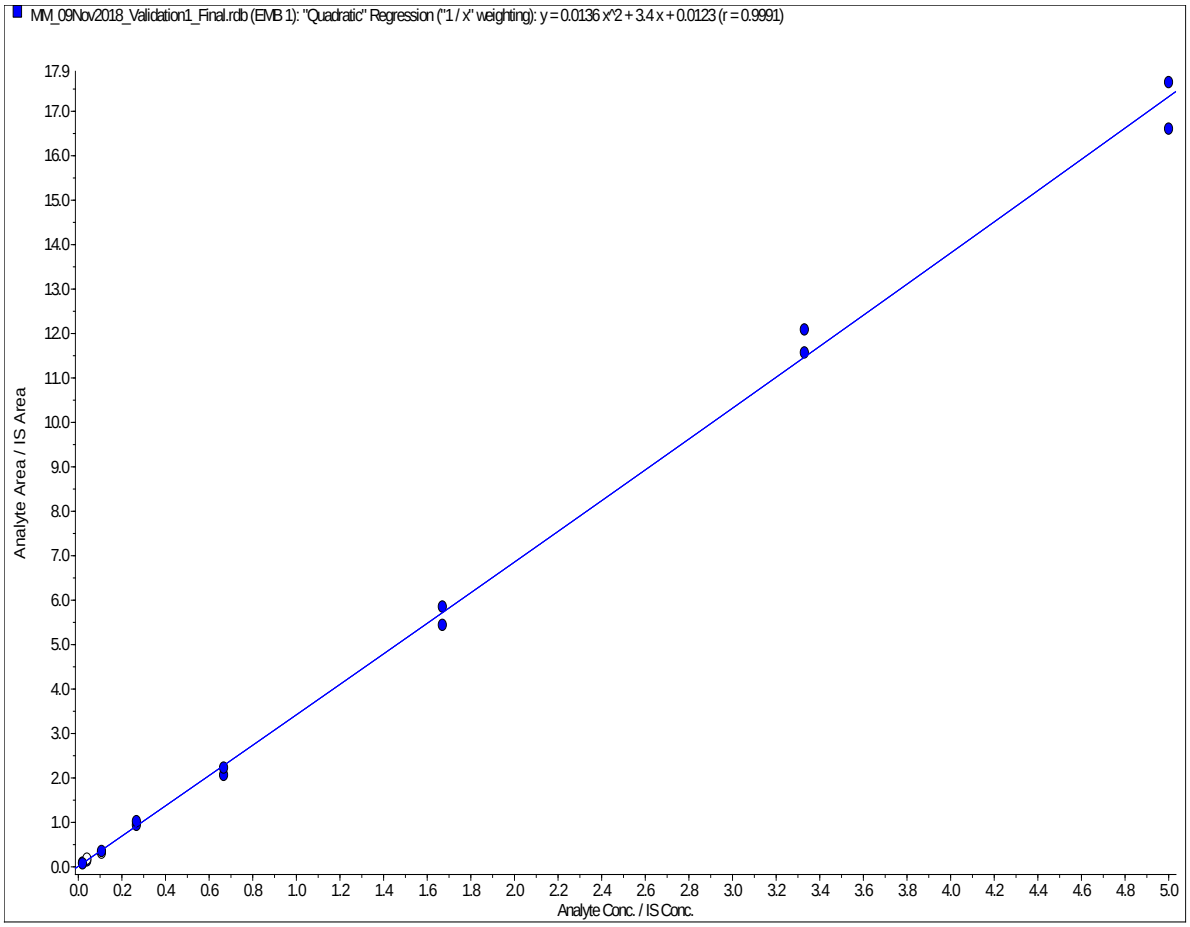

Figure 6.16: Representative calibration curve for EMB: Validation 1, Day 1

The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 6.87.

Table 6.87: Regression equation: EMB

Validation	**Quadratic Calibration Curve Parameters**			
Batch	**a**	**b**	**c**	**r**
1	0.0136	3.4	0.0123	0.9991

INH

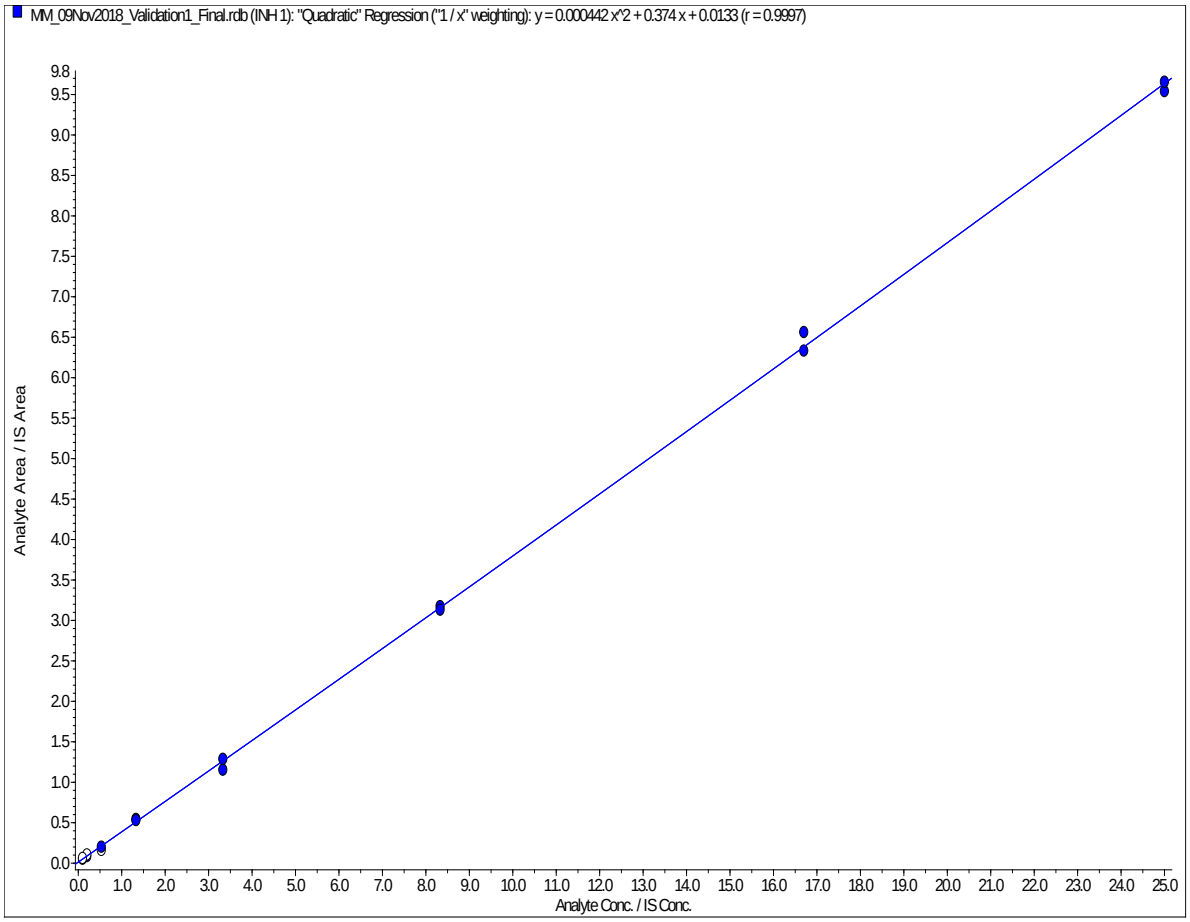

Figure 6.17: Representative calibration curve for Isoniazid: Validation 1, Day 1

The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 6.88.

Table 6.88: Regression equation: INH

Validation	**Quadratic Calibration Curve Parameters**			
Batch	**a**	**b**	**c**	**r**
1	0.000442	0.374	0.0133	0.9997

PZA

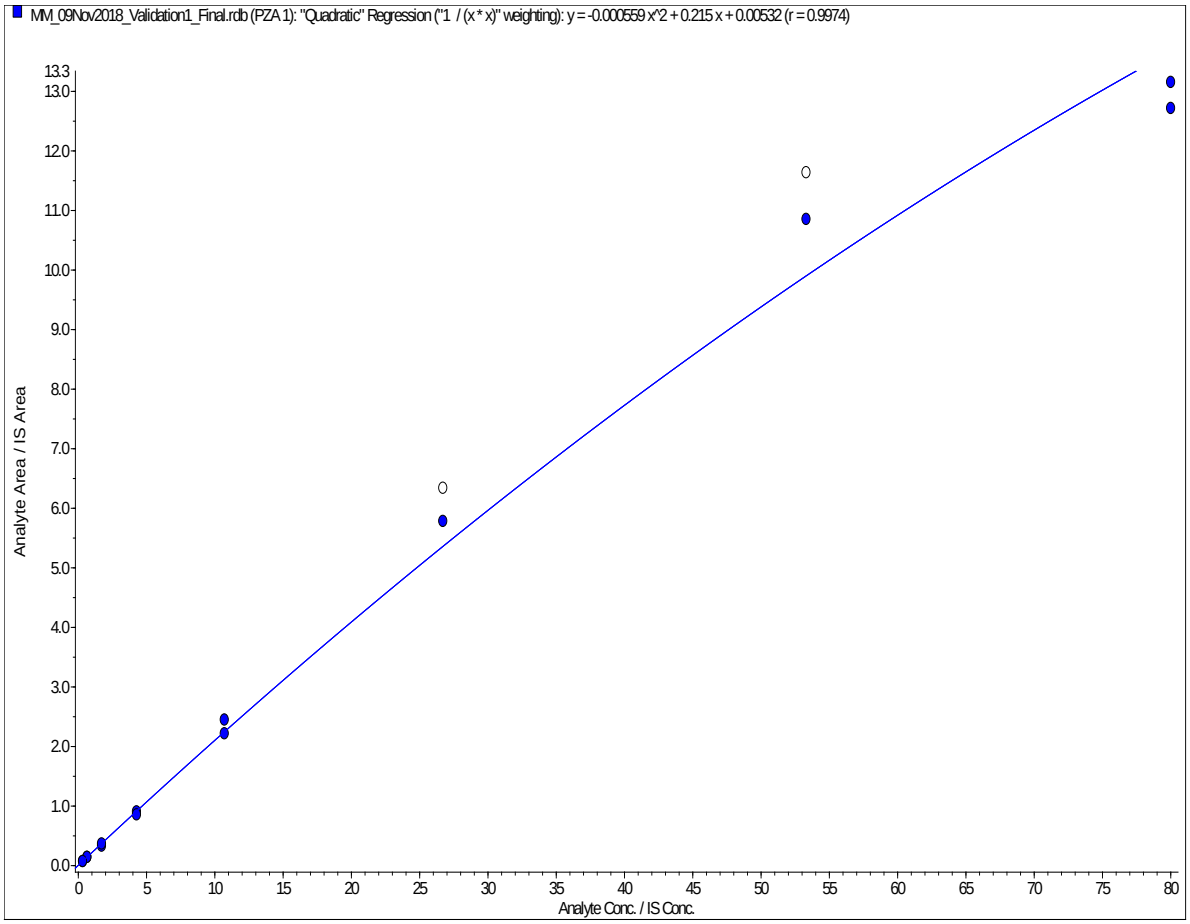

Figure 6.18: Representative calibration curve for PZA: Validation 1, Day 1

The regression equation used was Quadratic (weighted by $1/x^2$ concentration), $f(x) = a + bx + cx^2$, as presented in Table 6.89.

Table 6.89: Regression equation: PZA

Validation	**Quadratic Calibration Curve Parameters**			
Batch	**a**	**b**	**c**	**r**
1	-0.00056	0.215	0.00532	0.9974

RIF

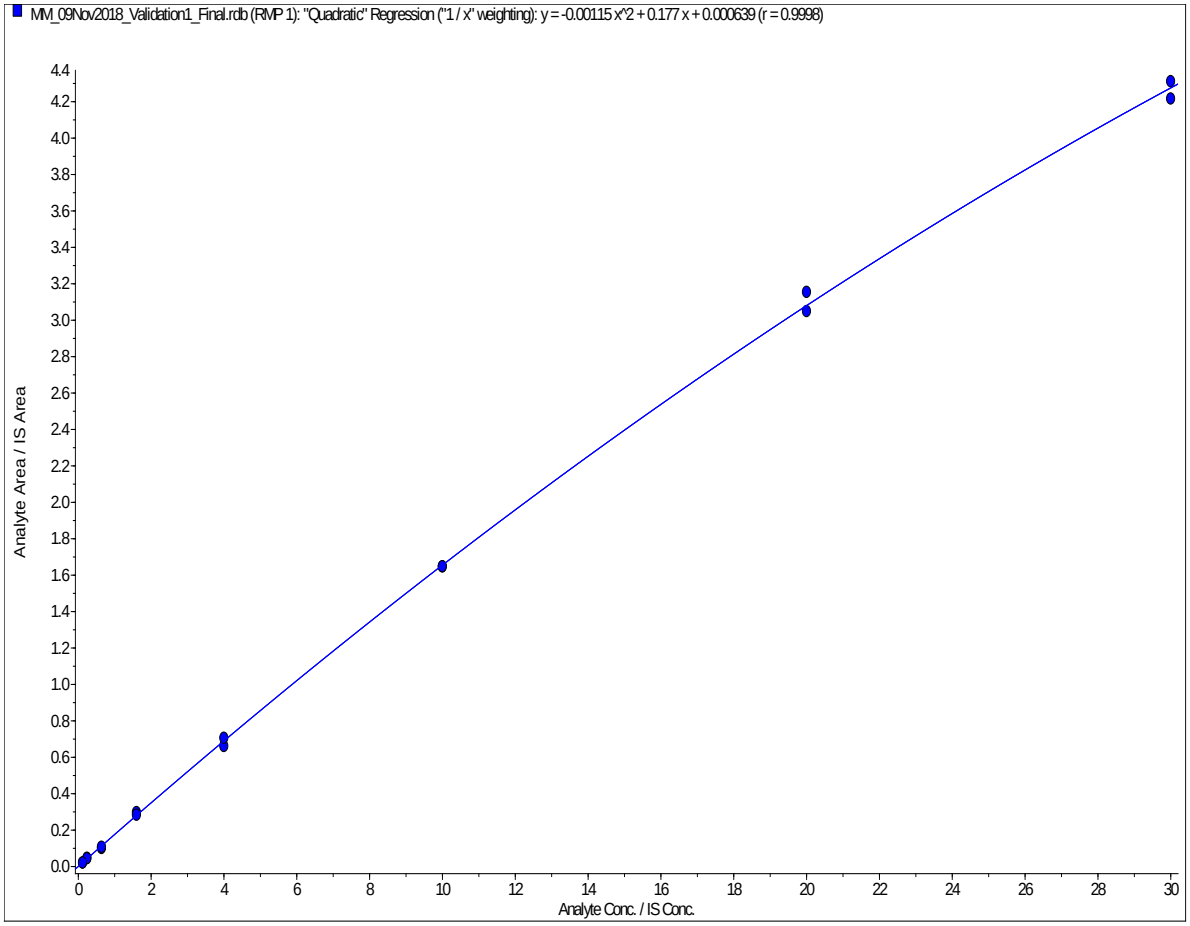

Figure 6.19: Representative calibration curve for Rifampicin: Validation 1, Day 1

The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 6.90.

Table 6.90: Regression equation: RIF

Validation Batch	**Quadratic Calibration Curve Parameters**			
	a	**b**	**c**	**r**
1	-0.00115	0.177	0.000639	0.9998

6.10. Carry over

In all analytical batches, carry over was assessed by injecting a double blank sample (containing no analyte or internal standard) immediately after the highest calibration standard. A blank sample, with IS and no analyte, was also included to determine possible contamination of the analyte by the IS. For all analytes, there was no significant carry over from the injection with the highest concentration; any peaks on the double blank sample were less than 20% of the peaks obtained at the LLOQ. No contamination of the analyte by the IS was observed.

Chapter 7 : Discussion

A method was developed and validated for the simultaneous quantification of first-line oral anti-TB drugs and two metabolites in human plasma. Although the analytes have widely varying physicochemical properties they were separated within 6 minutes using reverse-phase liquid chromatography as shown in Figure 6.7. As far as we know, one method has been developed to quantitate these 6 analytes (20). However, this method used structurally similar compounds as ISs whereas we used isotopically labelled ISs. The method by Song *et al.* used two ISs to cover the 6 analytes while the current method used an almost identical IS for each analyte, which could better compensate for matrix effects, process- and stability- related losses. The methods also had different calibration ranges for all analytes.

Although deuterated ISs were used for all analytes to compensate for matrix effects, the ISs themselves may affect the ionization of analytes and their ionization may also be affected by the corresponding analytes. However, this problem is not supposed to affect the robustness of the method since the internal standard concentrations will be constant in each analytical batch.

The method was accurate and reproducible, with accuracies and precisions less than 15% for all analytes. Analytes were stable on ice for at least 4 hours and samples could undergo three freeze-thaw cycles without negatively affecting the stability of analytes. Analysis of plasma samples with 2% haemolysed blood showed that the % Differences between haemolysed and non-haemolysed plasma were within 15% for three of the six analytes (AcINH, INH and RIF), hence 2% haemolysis does not negatively affect the accuracy for these analytes. The % Difference for desRIF, EMB and PZA was greater than 15% at the low concentrations

indicating that low concentrations of these analytes could not be reliably be measured in plasma samples that are 2% haemolysed. Previous validation reports indicate that 1% haemolysed plasma samples had no significant effect on the quantification of AcINH and INH (92) and EMB (90). However, unlike in this project, desRIF, and PZA were not significantly affected by 2% haemolysis (91, 93). Re-assessment of the effect of 2% haemolysis is therefore necessary.

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PZA showed instability on re-injection. However, a previous report showed that PZA could be reliably quantitated when reinjected after 24 hours (93). Therefore, the PZA data reported here will be re-investigated.

Future work

1. Due to matrix and time constraints, “fresh” vs “frozen” stability experiments still need to be done.
2. Re-injection stability of PZA showed contrasting results with previous reports therefore the PZA data reported here will be re-investigated.
3. The stability of desRIF and EMB in whole blood will be determined at a later stage.
4. Analysis of patient samples.

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APPENDIX 1: Human Research Ethics Approval Letter

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

15 June 2018

HREC REF: 372/2018

Dr L Wiesner
Pharmacology
K-floor, OMB

Dear Dr Wiesner

PROJECT TITLE: SIMULTANEOUS QUANTIFICATION OF FIRST-LINE ANTI-TUBERCULOSIS DRUGS AND METABOLITES IN HUMAN PLASMA (Masters Candidate - Ms M Mazanhanga)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 June 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

The HREC acknowledge that the student, Marian Mazanhanga will also be involved in this study.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC 372/2018